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REMARKS

Status of the claims

Claims 1-9, 11, 12, 15, 17-25, 34, and 36-43 are pending and under consideration in this application. All the pending claims stand rejected. After entry of the amendments made herein, claims 1, 2, 4-6, 8, 9, 11, 12, 15, 17-25, and 36-46 will be pending and under consideration in this application, claims 3 and 7 having been cancelled herein and claims 44-46 having been added herein. New claim 44 specifying that the targeting cells can be CD4+ T cells is supported by the specification (e.g., at page 12, lines 25-31). The teaching by the specification that CD4+ T cells can function as targeting cells was confirmed by the declaration of Daniel A. Vallera submitted March 27, 2002. Some of the alternative embodiments specified by original claim 5 have been deleted in claim 5 and included in new claims 45 and 46. None of the new claims and amendments made herein add new matter.

Telephone Interview

Applicants thank the Examiner and Examiner Amy Nelson for their helpfulness, patience, and courtesy in a telephone interview with Applicants' undersigned representative on May 25, 2004. Applicants are similarly grateful to the Examiner for her telephone call to Applicant's undersigned representative on May 27, 2004.

35 U.S.C. § 112, paragraph one, rejection

Claim 34 stands rejected on the ground that the specification allegedly does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with the claims.

From the comments on page 3, lines 9-15, of the Office Action, Applicants understand the Examiner's position to be that the specification enables administration by only intravenous injection. Applicants respectfully disagree with this position.

First, the specification teaches that administration of the targeting cells of the invention can be by both local and systemic routes (e.g., at page 36, line 31, to page 37, line 1). Such routes are well-known in the art and some are specifically mentioned in the specification (e.g., at page 34, lines 21-26). In addition, the declaration submitted on March 27, 2002, showed that intraperitoneal injection of the targeting cells had therapeutic efficacy against systemic leukemia. Given the efficacy of the "indirect" (i.e., intravenous and intraperitoneal) administrations described by the specification and the above-cited declaration, Applicants submit that one skilled in the art would expect that "direct" routes (such as intratumoral injection or direct application to a tumor bed after surgical excision of a tumor; e.g., see the specification at, for example, page 34, lines 21-26) would be even more effective and that other "indirect" administrations would likely be similarly effective.

From the comments on page 3, line 16, to page 4, line 17, of the Office Action, Applicants understand the Examiner's position to be that the specification is not enabling because it does not teach which of the receptors for the large number of molecules recited as being useful as targeting domains, other than IL-3 and IL-4, are particularly abundant on tumor cells versus by-stander normal cells. Applicants submit that, given the teaching of the specification (e.g., at page 19, line 6, to page 23, line 14; page 33, lines 8-18; and Example 6) and the state of the art (which is extremely high), practitioners in the field would be able, with no more than mere routine experimentation, to select a targeting molecule appropriate for a cancer of interest. Nevertheless, in order to expedite prosecution of the instant application, Applicants have limited claim 1, and thus also claims dependent on it (including claim 34), to specify that that the first member of the affinity pair (i.e., a targeting molecule) be a cytokine, a growth factor, or a colony stimulating factor. For consistency, claims 4-6, 9, and 38 have been similarly amended. This embodiment is supported by the specification (e.g., at page 19, lines 18-23) and adds no new matter.

The Examiner apparently believes that, because receptors for many of the targeting molecules of interest are expressed on by-stander normal cells, excessive toxicity would be render appropriate treatment methods unworkable. Applicants enclose copies of references

teaching the efficacy of *in vivo* treatment of appropriate cancers with immunotoxins such as those expressed by the targeting cells of the present invention (see below). As in all cancer therapies, there are indeed side effects of the treatments described in the enclosed references but these are, or are expected to be, controllable and/or transient. Using the methods of the instant invention in which relevant immunotoxins are focused on tumors by appropriate targeting T lymphocytes, such by-stander toxicity would be expected to be even less than that observed with treatment with the immunotoxins *per se*, as described in the enclosed references.

Thus, for example, the therapeutic efficacy, with controllable side effects, of immunotoxins containing interleukin (IL)-2 as the targeting molecule have been extensively described [see, example, Kreitman et al. (1992) Int. J. Immunopharmacol. 14(3):465-472 (page 468-469); LeMaistre (2000) Clinical Lymphoma 1(Suppl. 1):S37-S40 (whole article); Foss (2001) Annals of the NY Academy of Sciences 941:166-176 (whole article); copies enclosed as Exhibit A]. Indeed such an immunotoxin (marketed as ONTAK®) has been approved for treatment of patient with cutaneous T cell lymphoma [see Product Description enclosed as Exhibit B].

Kreitman et al. also describes research pointing to the usefulness of IL-6-containing immunotoxins [page 466-468].

There are many publications describing the usefulness of immunotoxins in which relevant targeting molecules (e.g., epidermal growth factors and transforming growth factors) bind to one or more classes of epidermal growth factor receptors; such receptors have been shown to be over-expressed in a variety of cancers [see, for example, Kreitman et al., page 466; and the following Abstracts enclosed as Exhibit C: LeMaistre et al. (1994) Breast Cancer Res. Treat 32(1):97-103; Yang et al. (1998) Clin. Cancer Res. 4(4):993-1004; Chandler et al. (1998) Int. J. Cancer 78(1):106-111; Psarras et al. (1998) Protein Eng. 11(12): 1285-1292; Yoon et al. (1999) Life Sci. 64(16): 1435-1445].

Moreover, the inventors have shown that an immunotoxin employing granulocyte-macrophage colony-stimulating factor (GM-CSF) as a targeting molecule, which killed myelomonocytic tumor cells, had little effect on early erythroid/myeloid stem cells and

multilineage progenitor cells, although it was active against committed myeloid progenitor cells [Chan et al. (1995) Blood 86(7):2732-2740 (see page 2738 column 1, paragraph 3, lines 12-18); copy enclosed as Exhibit D]. These findings indicated that treatment of a subject having cancer with an immunotoxin containing GM-CSF as a targeting domain would have an effect on normal myeloid cells in the subject but that such an effect would be transient.

Furthermore, recent studies have shown the usefulness of immunotoxins containing as targeting molecules IL-13 or urokinase-type plasminogen activator for treating glioblastomas [see, for Example, the following two Abstracts enclosed as Exhibit E: Li et al. (2002) Protein Eng. 15(5):419-427; Rustamzadeh et al. (2003) J. Neurooncol. 65(1):63-75].

In addition, Kreitman et al. refers to studies performed with analogous immunotoxins using insulin-like growth factor or acidic fibroblast growth factor as targeting molecules (page 466, column 1, paragraph 3).

In sum, the above references provide ample evidence of the efficacy and adequate safety of immunotoxins containing, as targeting a molecules, a wide variety of cytokines, growth factors, and colony stimulating factors and thus confirm that these molecules are indeed, as stated in the specification, useful for the purpose recited by the specification. Hence the pending claims are indeed enabled in their full scope by the specification.

Applicants will provide copies of additional relevant articles, articles referred to by Kreitman et al., or full articles corresponding to Abstracts submitted herewith should the Examiner so wish.

From the comments on page 4, lines 8-16, of the Office Action, Applicants understand the Examiner's position to be that the claims are inconsistent with specification because the claims specify that the targeting cells be T lymphocytes but the specification states that "the targeting cell preferably should not express a high level of receptors that bind targeting domain of the fusion protein. More preferably, the targeting cells should express no such receptors." Applicants respectfully disagree with this position.

First, Applicants point out that quoted statements merely state that the targeting cells preferably or more preferably express the relevant levels of relevant receptors. Thus, they do not

absolutely require that they express the stated receptor levels and thus the specification is not inconsistent with the claims. In this regard, Applicants submit that there are situations where it is not necessary, and indeed undesirable, that the targeting cells survive longer than necessary to produce a local level of immunotoxin sufficient to kill themselves.

In addition, Applicants submit those skilled in the art would know how to manipulate variables such as the level of receptors expressed by targeting cells of interest (e.g., by selecting clones of targeting cells expressing relatively low levels of the receptor), the level of immunotoxin produced by the targeting cells (e.g., by using regulatory elements of variable efficiency), or the structure of the immunotoxin. For example, in regard to the last variable, Kreitman et al. describes the work of others, which showed that by placing the toxic domain at the N-terminus of the targeting domain, and *vice versa*, it was possible to alter the potency of the resulting immunotoxin (page 466, column 1, last paragraph, and column 2, first paragraph). In addition, Kreitman et al. describes work showing that an immunotoxin containing IL-2 and one variant of *Pseudomonas* exotoxin (PE) as the toxic domain killed tumor cells and not normal (i.e., non-malignant) activated human lymphocytes; on the other hand, an IL-2-containing immunotoxin containing another variant of PE killed tumor cells as well activated human lymphocytes (page 468, column 2, paragraphs 2 and 3).

From the text on page 4, lines 16-21, of the Office Action, Applicants understand the Examiner to be confused as to how a targeting molecule and a targeted molecule in the instant invention can be an antigen and an antibody, respectively. Applicants draw the Examiner's attention to page 20, lines 8-15, of the specification where this apparent contradiction is explained. In brief, the antigens recognized by surface immunoglobulins (i.e., antibody molecules) of certain B cell lymphomas have been defined. Thus, such antigens could be used to target an appropriate toxins to relevant B cell lymphoma cells.

In light of the above considerations, Applicants respectfully request that the rejection under 35 U.S.C. § 112, paragraph 1, be withdrawn.

35 U.S.C. § 112, second paragraph rejections

Claims 36-38 stand rejected and claims 1-9, 11, 12, 15, 17-25, 34, and 36-43 are newly rejected as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter that Applicants regard as the invention.

With respect to the comments on page 5, lines 11-20, of the Office Action, Applicants understand the Examiner not to have appreciated the difference in the claim between the "cell population" and "cell preparation" as recited in claim 36. The term "cell population" refers to cells that have undergone the treatment recited in claim 36, i.e., cells after transfection or transduction with vector expressing an appropriate immunotoxin. On the other hand, the term "cell preparation" refers to the starting cells that are transfected or transduced in order to produce the "cell population". While Applicants believe claim 36 not to be indefinite, Applicants have significantly amended claim 36 in order to further clarify it.

Thus the term "preparation" in claim 36 has been replaced with the equivalent term "sample". In addition, other possibly confusing terms have been deleted or replaced and the final "wherein" clause has been greatly shortened by replacing the recitation of the result of the claimed method with a reference to claim 22.

From the text on page 5, line 21, to page 6, line 2, of the Office Action, Applicants understand the Examiner's position to be that the term "significant binding affinity" to be vague and indefinite. Applicants' undersigned representative pointed out the definition of the term in the specification (see page 2, lines 14-19) to the Examiner and Examiner Nelson during the telephone interview on May 25, 2004. Applicants' undersigned representative understands the rejection to be considered moot by both the Examiner and Examiner Nelson in view of the definition of the term in the specification.

In light of the above considerations, Applicants respectfully request that the rejection under 35 U.S.C. § 112, second paragraph, be withdrawn.

35 U.S.C. § 103(a) rejections

(a) Claims 1, 2, 4-6, 8, 9, 11, 12, 15, 17-25, and 36-42 stand rejected as allegedly being unpatentable over Paul et al. (the '387 patent), in view of Chan et al., Debinski et al., and Chen et al. Applicants respectfully traverse the rejection.

From the comments on page 6, line 25, to page 11, line 17, Applicants understand the Examiner's position to be that, in view of the combined cited art, the above-listed claims are obvious. Applicants disagree with this position.

Applicants point out that the arguments against obviousness in the present claims are mostly "lack of motivation to combine" arguments, i.e., arguments that the cited references provide no teaching, suggestion, or motivation to combine their respective teachings and thus render the invention obvious. The Examiner points out repeatedly in the Office Action that in Applicants' prior response that, while the rejection was based on a combination of references, Applicants had attacked the references individually (see, for example, pages 7, lines 6-11; page 12, lines 3-4; page 13, lines 20-22; and page 14, lines 15-20, of the Office Action). Applicants respectfully submit that, because Applicants' arguments are "lack of motivation to combine" arguments, it is necessary for Applicants to analyze each reference and, if appropriate, argue why it provides no motivation to combine.

In the telephone conversation with Applicants' undersigned representative on May 27, 2004, the Examiner indicated she had concluded that the '387 did not provide the necessary "motivation to combine" and that Applicants should focus their attention on the Chen et al. reference in arguing against the obviousness of the instant claims. Applicants again thank the Examiner for communicating this advice. Thus, in the arguments below, comments on the '387 patent will be relatively brief.

Claims 1, 2, 4-6, 8, 9, 11, 12, 15, 17-25, and 36-42

The Chen et al. reference describes the construction and use (*in vitro* and *in vivo*) of recombinant T cell tumor cells expressing an exogenous nucleotide sequence that encodes an immunotoxin composed of: (a) a targeting domain consisting of a single chain Fv antibody (sFv)

specific for HER-2, an antigen expressed on the surface of a variety of cells; and (b) a toxic domain consisting of truncated *Pseudomonas* exotoxin A (PEA).

Applicants point out that the T cell tumor cells used by the authors of Chen et al. for delivery of the above-described immunotoxin to nude mice had no demonstrated or inherent significant binding affinity for a cancer cell as required by instant claim 1. While the introductory paragraph of Chen et al. does refer to cytotoxic lymphocytes, it does so only in the context of: (i) describing the difficulty in obtaining tumor-specific lymphocytes; and (ii) describing the new class of cytotoxic lymphocytes described in the article. It is implicit from this text that this new class of cytotoxic lymphocyte is particularly desirable precisely because it avoids the problems of obtaining tumor-specific cytotoxic lymphocytes. Thus, not only does the Chen et al. reference not suggest the use of T cells with significant binding affinity for cancer cells, it recommends against their use. The reference teaches how to make T cells (lacking any defined specificity *per se*) into useful cytotoxic cells that are only specific for tumor cells of interest by virtue of the tumor-specific sFv targeting domain that targets the toxin (to which the sFv is bound) to the relevant tumor cells (e.g., Abstract, lines 9, 14, and 15; page 79, column 1, paragraph 2, lines 1-7). On the contrary, the targeting cells of the instant claims are unequivocally required, *per se*, to have specificity (significant binding affinity) for tumor (cancer) cells.

That Chen et al. lacks any motivation to use cancer-specific T lymphocytes is further indicated by the complete absence in the article of any disclosure, or even a suggestion of the desirability, of using the antigen specificity of T cells as an additional means of targeting a toxin to a cancer cell. The T cells of Chen et al. serve merely as "factories" of immunotoxin molecules which, by virtue of the tumor cell specificity of the sFv targeting domains, bind to appropriate tumor cells, which are then killed by the toxic moiety, i.e., the truncated PEA moiety.

The '387 patent, like many biotechnological patent documents and scientific articles, does mention TIL (tumor infiltrating lymphocytes). However, in that Chen et al. makes no mention of methods of improving the targeting of expression vectors to any of a variety of cells

(to which the invention the of the '387 patent is directed), it contains no motivation to combine its disclosure with that of the '387 patent.

As pointed out above, the Examiner has concluded that the '387 also lacks the motivation to combine its disclosure with Chen et al. (and the other cited art). Applicants agree with the Examiner. In that the '387 patent neither discloses or even suggests the use of TIL for delivering any molecule (let alone an immunotoxin) to any subject (let alone a subject with cancer), the '387 does indeed contain no motivation to combine its disclosure with that of Chen et al.

Moreover, in that neither Chan et al. nor Debinski et al. describe ways of improving expression vector uptake by cells or the use of recombinant immunotoxin-producing cells of any type, let alone T cells having significant binding affinity for cancer cells, neither reference provides the motivation to combine the disclosures of Chen et al. and the '387 patent.

Moreover, precisely because Chen et al. recommends against the use of tumor-specific lymphocytes for its purposes (see above), even if one ordinary skill in the art had been motivated by Chen et al., the '387 patent, Chan et al, and/or Debinski et al. to combine the disclosure of Chen et al. with that of the '387 patent, such an artisan would still not have considered replacing the tumor T cells employed by Chen et al. with TIL.

The Examiner cites *In re Kerkhoven*, 205 USPQ 1069 (CCPA 1980) for the proposition that "it is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose in order to produce a third composition that is to be used for the very same purpose" (Office Action, page 11, lines 5-15). From this holding, she concludes that it would have been *prima facie* obvious to one of ordinary skill in the art to combine tumor specific CTL and immunotoxins to generate a new composition for the treatment of cancer with a reasonable expectation of success (Office Action, page 11, lines 10-15). Applicants strongly disagree with this conclusion. Applicants respectfully submit that it is a very different matter to combine, on one hand, "two conventional spray-dried detergents" (as in *In re Kerkhoven*, 205 USPQ 1069, 1072) and to combine an immunotoxin and CTL. Contrary to the situation in *In re Kerkhoven*, immunotoxins and CTL are entirely different agents, each acting by a very different

mechanism and it is far from clear that one of skill in the art would have considered combining them.

In addition, Applicants submit that even if the prior existence of the two anti-tumor agents were to have suggested a simple combined administration of CTL and immunotoxins to a subject with cancer, such a suggestion would have constituted a mere invitation to try with not the least assurance of success. Moreover, in the present invention in which administered T cells (e.g., CTL) are transformed with an expression vector encoding an immunotoxin, multiple steps in addition to merely "combining" are involved, each additional step having its inherent problems. Applicants respectfully submit that, if one of skill in the art at the priority date of instant application had considered making and administering the claimed targeting cells, the chance of success would have been considered by such an artisan to be orders of magnitude less than that of a simple combined administration of CTL and immunotoxins.

Finally, contrary to the case in *In re Kerkhoven* (295 USPQ 1069, 1072), the present combination (CTL transformed expressing recombinant immunotoxin) was shown to function significantly better than a single agent on its own (CTL alone) (see Example 6 in the present specification).

Claims 38-42

In the immunotoxins encoded by the viral vectors of the instant claims, the targeting molecules are cytokines, growth factors, or colony-stimulating factors. On the other hand those disclosed by Chen et al. are sFv fragments. For the reasons given above, neither Chen et al. nor the '387 patent contain motivation to combine their respective disclosures. Moreover, even if they did provide such motivation, in that there is no disclosure in the '387 patent of an immunotoxin of any sort, let alone one in which the targeting domain is a cytokine, growth factor, or colony-stimulating factor, the '387 patent adds nothing to the disclosure of Chen et al. in terms of what moieties can be used as targeting domains in immunotoxins.

Debinski et al. and Chan et al. disclose immunotoxins containing cytokines (IL-4 and IL-3, respectively) as targeting domains. There is no disclosure, or even a suggestion in Chen et al.

of using anything other than a sFv, let alone a cytokine, a growth factor, or a colony-stimulating factor as a targeting domain. Thus, Chen et al. provides no motivation to combine its disclosure with either Debinski et al. or Chan et al. and thus to make a retroviral immunotoxin in which targeting domain is a cytokine, growth factor, or colony-stimulating factor.

Debinski et al. and Chan et al. describe plasmid vectors only for use in bacterial production of immunotoxic proteins, which are then used for *in vitro* experiments only (Debinski et al.) or for both *in vitro* and *in vivo* experiments (Chan et al.). Neither Debinski et al. nor Chan et al. mention, or even suggest, the use of any vectors, let alone viral vectors, to transform mammalian cells for any purpose, let alone for the purpose of making immunotoxin "factories". Thus, neither reference discloses or even suggests the use of a viral vector. In addition, neither of the two references refer to, or even suggest the desirability of using, any molecules other than IL-3 or IL-4, let alone antibody fragments such as sFv, as targeting domains for immunotoxins. Thus, neither of Debinski et al. and Chan et al. contain the necessary motivation to combine their respective disclosure with that of the Chen et al. and thus to make viral vectors of the instant claims.

In that the '387 patent does not mention immunotoxins of any sort, let alone those in which in the targeting domain is a sFv fragment (as in Chen et al.) or a cytokine (as in Debinski et al. and Chan et al.), it also lacks the motivation to combine the disclosure of Chen et al. with that Debinski et al. and/or Chan et al.

Thus, none of the cited references, considered alone or in combination, disclose or even suggest the invention of the instant claims.

(b) Claims 3 and 7 stand rejected as allegedly being unpatentable over Paul et al. (the '387 patent), in view of Chan et al., Debinski et al., and Chen et al., and further in view of Cochlovius et al.

The rejection is moot in light of the cancellation of claims 3 and 7.

(c) Claim 43 is rejected as allegedly being unpatentable over Paul et al. (the '387 patent), in view of Chan et al., Debinski et al., and Chen et al., and further in view of Clay et al. or Buchsbaum et al. (the '329 patent). Applicants respectfully traverse the rejection.

From the comments on page 13, line 17, to page 14, line 20, of the Office Action, Applicants understand the Examiner's position to be that, in mentioning viral vectors, Clay et al. or the '329 patent provide what is missing from the other cited art and thus, in combination with that art, render claim 43 obvious. Applicants disagree with this position. While both references list various viral vectors, because of the purposes for which the authors of the two references used the relevant viral vectors, the two references provided no motivation to combine their disclosures with those of the other cited references, which similarly provided no motivation to combine their disclosure with that of Clay et al. and/or the '329 patent.

For the reasons given above, none of Chen et al., the '387 patent, Chan et al., and Debinski et al. contain the requisite motivation to combine their respective disclosures.

While Clay et al. provides a general discussion of a number of viral vectors (pages 4 and 5), the reference particularly describes experiments with retroviruses expressing only T cell receptor genes and designed to generate CTL with specificity for a melanoma cells (e.g., Abstract). There is no disclosure, or even suggestion, in Clay et al. of retroviruses (or any of the other viral vectors referred to) expressing exogenous tumor cytotoxic molecules of any sort, let alone the immunotoxins of Chan et al. and Debinski et al. Thus, Clay et al. contains no motivation to combine its disclosure with that of Chan et al. or Debinski et al. and to make viral vectors expressing their respective immunotoxins. In addition, since Chan et al. and Debinski et al. make no mention at all of transforming mammalian cells and thus provide no reason to use any vectors other than plasmid vectors (see above), they provide no motivation to combine their disclosure with that of Clay et al.

In that neither Chen et al. nor Clay et al. discloses or even suggests the use of non-antibody targeting domains in immunotoxins, the combination of the two references fails to render obvious the viral vectors of the instant invention. The same is true for the combination of the '387 patent and Clay et al.

The '329 patent describes the production and use of radiolabeled immunotoxins containing a cytokine, a toxin, and a radionuclide (e.g., Abstract). However, the '329 patent does not teach the use of viral vectors expressing any immunotoxin, let alone those of Chan et al. or Debinski et al. The only context in which the '329 patent mentions viral vectors (adenoviral vectors) is for creating experimental recombinant cancer cells expressing: (a) an experimental exogenous antigen of interest (i.e., carcinoembryonic antigen; CEA) (columns 19-20); or (b) an exogenous cytokine (GM-CSF, EGF, or IL-4) receptor to which the reference's radiolabeled immunotoxins containing corresponding cyokine targeting domains can bind (columns 35-36).

Since in terms of immunotoxin delivery the '329 patent only teaches direct administration of radiolabeled protein immunotoxins *per se*, it does not contemplate transforming mammalian cells, *in vitro* or *in vivo*, with an immunotoxin-encoding nucleic acid sequence; hence, not surprisingly, it neither discloses nor even suggests an adenoviral vector expressing any immunotoxin, let alone those it discloses itself or those of Chan et al. or Debinski et al. Chan et al. and Debinski et al. are similarly only concerned with administering protein immunotoxins *per se* to subjects. None of the three references even suggests a reason to transform a mammalian cell to express any immunotoxin. Thus, even if one or more of the references contained the necessary motivation to combine their respective disclosures, no such combination would persuade one of ordinary skill in the art to make retroviral vectors encoding the immunotoxins encoded by the present claims.

Similarly, in that the '329 contains no suggestion of making an adenoviral vector encoding anything other than CEA or the above-recited cytokine receptors, a combination of the '329 patent and the Chen et al. reference would not motivate one skilled in the art to make an adenoviral vector encoding the immunotoxin disclosed by Chen et al. Moreover, even if such an artisan were so motivated, the resulting relevant immunotoxin would be one not specified by the instant claims, i.e., it would contain a sFv, and not a cytokine, a growth factor, or a colony-stimulating factor, as a targeting domain.

The '387 patent makes no mention of any immunotoxins, let alone those of the '329 patent, and the '329 patent makes no mention of adenoviral vectors encoding anything other than

CEA or a cytokine receptor (see above). Thus, even if the two references contained the motivation to combine their respective disclosures, doing so would not suggest to one of ordinary skill in the art an adenoviral vector encoding an immunotoxin specified by the instant claims.

In light of the above considerations, none of the cited references, singly or in combination, render claims 43 obvious.

In view of the above factors, Applicants respectfully request that the rejections under 35 U.S.C. §103(a) be withdrawn.

CONCLUSION

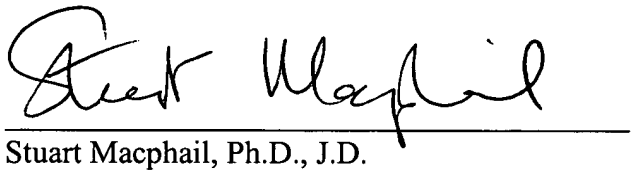
In summary, for the reasons set forth above, Applicants maintain that all of the pending claims patentably define the invention. Applicants request that the Examiner reconsider the rejections as set forth in the Office Action and permit the pending claims to pass to allowance.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicants' undersigned representative can be reached at the telephone number listed below.

Enclosed is a petition for an automatic extension of time and check in payment of the extension of time. Please apply any additional charges or credits to Deposit Account No. 06-1050, referencing Attorney Docket No. 11983-004001.

Respectfully submitted,

Date: 6/1/04



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TARGETING GROWTH FACTOR RECEPTORS WITH FUSION TOXINS

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Abstract — Recombinant toxins which bind to growth factor receptors have been prepared and used to kill cells responsible for malignant or autoimmune disease. Our strategy has been to genetically fuse ligands to different forms of *Pseudomonas* exotoxin which due to mutations or deletions do not bind to normal cells. The resulting recombinant chimeric toxins, in concentrations often less than 1 ng/ml, selectively kill cells expressing the appropriate growth factor receptor. The ligand may be a growth factor, such as transforming growth factor alpha (TGFα), interleukin 6 (IL6) or interleukin 2 (IL2), or single chain antigen binding proteins, such as the variable heavy and light regions of the monoclonal antibody anti-Tac. These chimeric toxins kill not only established cell lines but also fresh tumor cells from patients and display anti-tumor activity toward human malignant tumors in nude mice. While clinical trials are beginning with some of these agents, work continues to improve the effectiveness of recombinant chimeric toxins, and to widen the scope of disorders which might be treated by this approach.

The current established therapy of systemic malignant and autoimmune disease relies on immunosuppressive or cytotoxic chemotherapy which generally kills target cells because of biochemical differences between abnormal and normal cells. Often these differences are insufficient to effect cures without excessive toxicity. Hormonal therapy of malignant disease takes advantage of receptors preferentially expressed by tumor cells, but treatment with ligands alone does not generally kill the target cells. An evolving alternative is to design chimeric toxins composed of a growth factor, which will bind to a cell, and a toxin which after internalization will kill the cell.

In contrast to chemotherapy, the number of molecules of chimeric toxin allowed to internalize is regulated by the number of receptors, and is relatively low. Because of this, the toxin portion must be potent. Commonly used toxins include those that enzymatically inactive ribosomes (ricin, abrin, pokeweed anti-viral protein, saporin and gelonin) or ADP-ribosylate elongation factor-2 (Pastan *et al.*, 1986). Toxins in the latter group are bacterial in origin and include diphtheria toxin (DT) and *Pseudomonas* exotoxin (PE). We have focused on PE, since humans rarely carry antibodies against this toxin.

STRUCTURE AND FUNCTION OF PE

Pseudomonas endotoxin is a single chain 66 kd polypeptide composed of 613 amino acids. X-ray crystallographic and deletional analyses have established structural domains responsible for the different toxin functions (Allured *et al.*, 1986; Hwang *et al.*, 1987) (Fig. 1). Domain Ia, composed of amino acids 1–252, binds to the PE receptor which is present on most normal animal cells. At the carboxyl end, amino acids 400–613 contain the enzymatic portion which ADP-ribosylates EF-2. In the middle, amino acids 253–364 are contained in domain II, which is responsible for translocating a 37 kd fragment containing domain III and part of domain II into the cytosol. The 37 kd fragment is formed when the toxin is proteolytically cleaved near Arg-276, and the disulfide bond linking Cys-265 and Cys-287 is reduced (Ogata *et al.*, 1990). The function of amino acids 365–400, which make up domain Ib, is unknown, but amino acids 365–380 have been removed from several different fusion toxins without altering cytotoxicity (Siegall *et al.*, 1989a). Because the ADP-ribosylation step is catalytic, the toxin is very potent, and microinjection studies have shown that one toxin molecule is sufficient to kill a cell (unpublished data). However, because many toxin

molecules become degraded in lysosomes before being translocated to the cytosol, hundreds to thousands of toxin molecules must bind and internalize in order for the cell to die.

To make recombinant fusion toxins, we have constructed plasmids containing the T7 promoter (Studier & Moffatt, 1986). Expression, by lactose derepression, of the desired protein can be induced in *Escherichia coli* cells carrying these plasmids. The chimeric toxins discussed here are purified from the insoluble fraction by renaturation, followed by several chromatography steps (Chaudhary *et al.*, 1990a).

To make a useful chimeric toxin which would specifically kill target cells, we must prevent PE from binding to normal cells. One approach is to remove domain Ia, which results in a 40 kd protein referred to as PE40 (Fig. 2). The second is to mutate each of four basic amino acids within domain Ia to glutamate (Fig. 2), which results in a 66 kd molecule called PE^{4E} (Chaudhary *et al.*, 1990c). Neither PE40 nor PE^{4E} can bind to the PE receptor. Fusion toxins cytotoxic for cells expressing growth factor receptors have been produced by genetically fusing ligands such as interleukin 2 (IL2), interleukin 4 (IL4), interleukin 6 (IL6), transforming growth factor alpha (TGFα), insulin-like growth factor I (IGF-I), and acidic fibroblast growth factor (aFGF) to PE40 and/or PE^{4E} (Lorberboum-Galski *et al.*, 1988a, 1990; Ogata *et al.*, 1989; Siegall *et al.*, 1988, 1989b, 1990a, 1991a; Prior *et al.*, 1991). This discussion will focus on chimeric toxins cytotoxic against cells expressing epidermal growth factor (EGF), IL6, or IL2 receptors (Fig. 1).

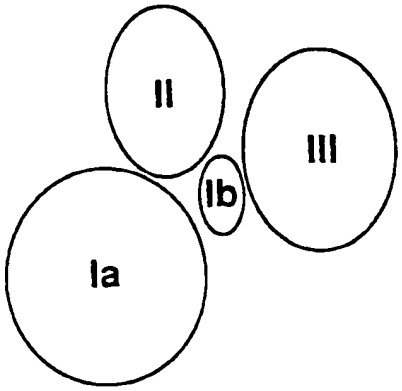

Fig. 1. Diagrammatic model of *Pseudomonas* exotoxin showing domains Ia,II,Ib, and III (from Pastan & FitzGerald, 1989).

TGFα-TOXINS AGAINST THE EGF RECEPTOR

TGFα, which is similar to EGF, appears to be involved in the growth and maintenance of many solid tumors, including those of lung, breast, head and neck, prostate, brain, hepatic, bladder, endometrial, renal and gastrointestinal origin (Lau *et al.*, 1988; Real *et al.*, 1986; Hendler & Ozanne, 1986; Jones *et al.*, 1990; Reynolds *et al.*, 1990; Wilding *et al.*, 1989; Scambia *et al.*, 1991). The first chimeric toxin using PE, PE40–TGFα, was made by genetically fusing TGFα to the carboxyl terminus of PE40 (Fig. 2) (Chaudhary *et al.*, 1987). PE40–TGFα could not be completely purified, and it was less cytotoxic to EGF receptor expressing epidermoid cell lines A431 and KB than was a chemical conjugate made with PE and EGF.

A significantly improved TGFα-toxin, TGFα–PE40, was produced when TGFα was fused to the amino terminus of PE40 (Fig. 2) (Siegall *et al.*, 1988). TGFα–PE40 was over 30-fold more cytotoxic to KB and A431 cells than was PE40–TGFα, and could be purified to near-homogeneity (Siegall *et al.*, 1989b). Probably because of the presence of EGF receptors on normal liver cells, the lethal dose (LD_{50}) by intraperitoneal (i.p.) injection of TGFα–PE40 is 1–2 μg in a mouse, which is nearly 25-fold lower than that of PE40. Nevertheless, therapeutic trials of TGFα–PE40 or related derivatives in nude mice carrying human epidermoid, hepatocellular, prostatic, or colon carcinoma have demonstrated a therapeutic window for its anti-tumor activity (Pai *et al.*, 1991; Edwards *et al.*, 1989; Heimbrook *et al.*, 1990; and unpublished data). A derivative of TGFα–PE40 without disulfide bonds in PE40 (TGFα–PE40δcys) (Heimbrook *et al.*, 1990) is currently being prepared for a clinical trial in bladder cancer.

IL6 TOXINS

Since it was found that IL6 receptors (IL6R) are expressed on human myeloma cells and various tumor cell lines (Kawano *et al.*, 1988; Taga *et al.*, 1987), we constructed the chimeric toxin IL6–PE40 (Siegall *et al.*, 1988) in which IL6 is fused to the amino terminus of PE40 (Fig. 2). We found IL6–PE40 to be cytotoxic not only toward human myeloma cell lines, but also toward hepatocellular

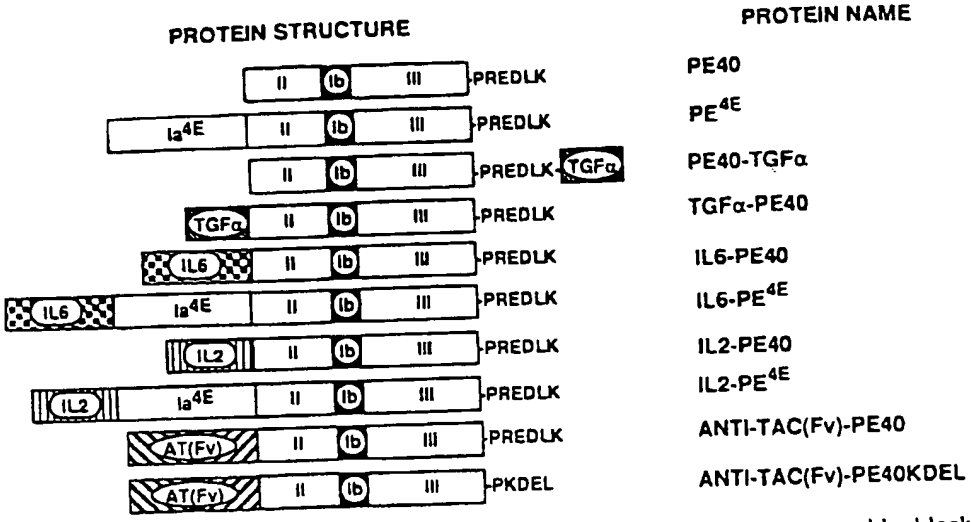

Fig. 2. Schematic models of fusion toxins discussed. Ligands and toxin domains are represented by blocks. The block for domain III contains only amino acids 400 – 607, so that differences in the carboxy termini of the fusion toxins can be specified.

Table 1. Cytotoxicity of IL6 – PE40 and IL6 – PE^{4E} against cell lines (Siegall *et al.*, 1990a,c)

Cell line	Type	ID_{50} (ng/ml)	
		IL6 – PE40	IL6 – PE^{4E}
U266	Myeloma	8 – 15	0.9 – 1.5
H929	Myeloma	8 – 12	1.5 – 3
PLC/PRF/5	Hepatoma	5 – 7	1.5 – 2
HEP3B	Hepatoma	18 – 30	40 – 50
HEPG2	Hepatoma	450	70
A431	Epidermoid carcinoma	> 625	8
LNCaP	Prostate carcinoma	9	1.7
DU145	Prostate carcinoma	> 1000	40

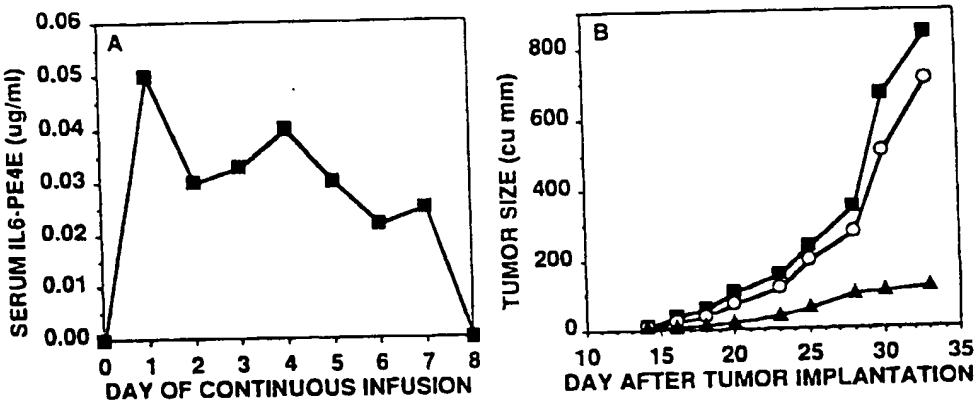

Fig. 3. By continuous infusion of IL6 – PE^{4E} 250 μg/kg/d × 7 days via an ALZA pump placed into the i.p. cavity of nude mice, we were able to detect significant serum levels measured by cytotoxicity assay of serum samples. Nude mice were injected with 1 × 10^7 PLC/PRF/5 cells subcutaneously and after 14 days developed tumors. The mice were tested with either IL6 – PE^{4E} 350 μg/kg/d × 7d IP (▲), or an equal dose of an ADP-ribosylation deficient IL6 – PE^{4E} mutant (○), or were untreated (■) (taken from Siegall *et al.*, 1991b).

Table 2. Activity of anti-Tac and IL2-toxins against human malignant cells (Kreitman *et al.*, 1990)

No.	Diagnosis	ID_{50}, ng/ml		
		Anti-Tac(Fv) – PE40	IL2 – PE40	IL2 – PE^{4E}
1	ATL	4	> 1000	250
2	ATL	16	> 1000	610
3	ATL	7.5	> 1000	1000
4	ATL	1.6		
5	ATL	10		
6	ATL	6		
7	ATL, nonleukemic	> 1000	> 1000	> 1000
8	ATL, nonleukemic	> 1000		
9	T-cell lymphoma	170		
10	Childhood ALL	> 1000		
11	Normal	> 1000	> 1000	> 1000
12	Normal	> 1000	> 1000	> 1000
13	HUT 102	0.2	17	0.8

carcinoma lines (Siegall *et al.*, 1990b). We also made IL6 – PE^{4E}, which contains the ligand fused to the amino terminus of PE^{4E} (Fig. 2). IL6 – PE^{4E} was not only more cytotoxic than IL6 – PE40 toward myeloma and hepatocellular carcinoma cell lines, but unlike IL6 – PE40 showed cytotoxicity toward human epidermoid and several prostate carcinoma lines (Table 1) (Siegall *et al.*, 1990a,c).

Because hepatocytes express IL6R (Bauer *et al.*, 1989), we wished to test whether we could safely administer enough IL6 – PE^{4E} to an animal to cause an anti-tumor effect. For our animal model, we used mice, since murine IL6R binds human IL6. We treated nude mice implanted with the human hepatocellular carcinoma line PLC/PRF/5 (Siegall *et al.*, 1991b). By administering the chimeric toxin by continuous infusion for one week from pumps implanted i.p., we were able to sustain serum IL6 – PE^{4E} levels between 0.025 and 0.05 μg/ml [Fig. 3(A)] and to inhibit the growth of tumor in treated animals [Fig. 3(B)]. This suggested that a therapeutic window for IL6 – PE^{4E} treatment might exist.

Since human myeloma cells have fewer IL6R per cell than myeloma lines (Kawano *et al.*, 1988) and might internalize toxin at lower rates than cell lines, we decided to test IL6 – PE^{4E} against myeloma cells obtained directly from patients. We tested Ficoll-purified bone marrow mononuclear cells, obtained from multiple myeloma patients, which contained mostly malignant cells. Without toxin, the cells incorporated [^{3}H]-leucine into protein quite well, even after 2 – 3 days in culture, and 6 of 10 samples were very sensitive to IL6 – PE^{4E}. Further studies on cells from myeloma patients are in progress. At this time IL6 – PE^{4E} is being considered for a clinical trial for treating multiple myeloma, either in phase I *in vivo* treatment or *ex vivo* purging of myeloma cells in autologous transplant protocols. We are also investigating the utility of IL6 – PE^{4E} in treating other malignancies.

IL2 RECEPTOR TOXINS

To make a chimeric toxin which could kill IL2 receptor bearing cells, we attached IL2 to the amino terminus of PE40 (Fig. 2) (Lorberboum-Galski *et al.*, 1988a). The resulting chimera, IL2 – PE40, was cytotoxic toward cells expressing IL2 receptors, and prevented growth of an IL2 receptor expressing tumor in mice (Kozak *et al.*, 1990). IL2 – PE40 was also cytotoxic to cell lines expressing either the p55 or the p75 component of the IL2 receptor (Lorberboum-Galski *et al.*, 1988b). Moreover, IL2 – PE40 was cytotoxic toward murine activated T-cells (Ogata *et al.*, 1988), and showed activity against adjuvant-induced arthritis in rats (Case *et al.*, 1989; Lorberboum-Galski *et al.*, 1991) and cardiac allograft rejection in mice (Lorberboum-Galski *et al.*, 1989). However, IL2 – PE40 did not kill activated human lymphocytes.

To make a more cytotoxic IL2-toxin, we placed IL2 at the amino terminus of PE^{4E} (Fig. 2). IL2 – PE^{4E} was not only more cytotoxic than IL2 – PE40 against several cell lines, but was also active against human activated lymphocytes (Lorberboum-Galski *et al.*, 1990). IL2 – PE^{4E} at concentrations of 5 – 50 ng/ml inhibited protein synthesis in human or monkey lymphocytes activated either by

mixed lymphocyte reaction (MLR) or phytohemaglutinin (PHA). However, a more active molecule was desirable.

To make an even more cytotoxic molecule toward IL2 receptor bearing cells, we decided to target the p55 component of the IL2 receptor, which on many cell lines outnumbers p75 by over 10-fold (Chaudhary *et al.*, 1989). Because IL2 has a relatively low affinity for p55 ($Kd \sim 10^{-8}$ M), we decided to use the monoclonal antibody anti-Tac, which has higher a affinity for the p55 (Tac) antigen ($Kd \sim 10^{-9}$ M) (Robb *et al.*, 1984). When anti-Tac was chemically conjugated to PE40, an immunotoxin was produced which inhibited protein synthesis in the adult T-cell leukemia line HUT 102 with an ID_{50} of 13 ng/ml (Kondo *et al.*, 1988; FitzGerald *et al.*, 1984). To make an immunotoxin which would not be subject to partial inactivation by chemical coupling and would contain the ligand only at the amino terminus of PE40, we made anti-Tac(Fv) – PE40, which is a recombinant fusion of the variable domains of anti-Tac with PE40 (Chaudhary *et al.*, 1989). The ligand was a single-chain antibody composed of the variable heavy (V_H) domain of anti-Tac fused to the variable light (V_L) domain through a 15 amino acid linker (Fig. 2).

Anti-Tac(Fv) – PE40 was at least 10-fold more cytotoxic toward target cells than either IL2-toxins or anti-Tac chemical conjugates. Against HUT 102 cells, anti-Tac(Fv) – PE40 inhibited protein synthesis with an ID_{50} of 0.15 ng/ml compared with 2.0 ng/ml with IL2 – PE^{4E} (Lorberboum-Galski *et al.*, 1990; Chaudhary *et al.*, 1989). Anti-Tac(Fv) – PE40 was over 200-fold more cytotoxic than IL2 – PE^{4E} against the human MT-1 line, which expresses only p55 and hence no high affinity IL2 receptors. Anti-Tac (Fv) – PE40 was also more cytotoxic than IL2 – PE^{4E} against activated T-lymphocytes (Batra *et al.*, 1990), being over 50-fold more cytotoxic in the PHA assay, and over 200-fold more cytotoxic in the MLR assay. Moreover, the LD_{50} of anti-Tac(Fv) – PE40 in mice was 10 – 25 μg (Batra *et al.*, 1990), the same as IL2 – PE^{4E} (Lorberboum-Galski *et al.*, 1990). We have been able to achieve serum levels of anti-Tac(Fv) – PE40 exceeding 1500 ng/ml in mice (Batra *et al.*, 1990) and 400 ng/ml in a monkey without apparent toxicity (Kreitman, Parenteau, FitzGerald, Waldmann and Pastan, unpublished data). Clearly from a therapeutic standpoint, anti-Tac(Fv) – PE40 appears superior to the IL2 fusion toxins.

To determine if anti-Tac(Fv) – PE40 would not only kill cell lines and activated T-lymphocytes but also malignant cells directly from patients, we tested the recombinant immunotoxin against peripheral blood mononuclear cells (PBMCs) from patients with ATL (Kreitman *et al.*, 1990). The PBMCs were obtained after Ficoll centrifugation of venous blood and were placed into culture with toxins or controls for 16 h. As with assays on cell lines, protein synthesis inhibition, an early indicator of cell death, was assessed by measuring [^{3}H]-leucine incorporation into protein. The PBMCs of each of six ATL patients (with lymphocyte Tac positivity ≥40%) were very sensitive to anti-Tac(Fv) – PE40, with ID_{50}s of 1.6 – 16 ng/ml (Table 2). In contrast, the PBMCs from normal donors, or normal PBMCs from ATL patients without blood involvement, were resistant. IL2 – PE^{4E} was at least 50-fold less cytotoxic than anti-Tac(Fv) – PE40 against the malignant cells. The ATL cells were resistant to anti-Tac, or an anti-Tac(Fv) – PE40 mutant deficient in ADP-ribosylation activity, indicating that cytotoxicity caused by anti-Tac(Fv) – PE40 toward the patient cells required not only immunotoxin binding, but internalization and ADP-ribosylation activity as well.

Recently, we have improved the cytotoxicity of anti-Tac(Fv) – PE40 by changing the carboxyl terminus from -REDLK to -KDEL (Fig. 2) (Seetharam *et al.*, 1991). The last five amino acids of PE, which are required for cytotoxicity but not ADP-ribosylation, probably facilitate internalization, processing within the endocytic vesicle, or translocation into the cytosol (Chaudhary *et al.*, 1990b). Anti-Tac(Fv) – PE40KDEL was several-fold more cytotoxic than anti-Tac(Fv) – PE40 against cell lines such as HUT 102, Cr II.2, and ELT5 (Seetharam *et al.*, 1991). Recently, we have found that anti-Tac(Fv) – PE40KDEL is 10-fold more cytotoxic than anti-Tac(Fv) – PE40 against MT-1 cells, and is several-fold more cytotoxic toward activated human lymphocytes and ATL cells. We are currently testing anti-Tac(Fv) – PE40 and derivatives in primate models of allograft rejection.

SUMMARY

We have made use of growth factor receptors expressed by cells causing malignant and autoimmune disease to target chimeric fusion toxins to those cells. We believe this may be an effective form of therapy, especially if the target cells require the growth factor to survive. For example, if a malignant cell becomes hormone-independent because of autocrine hormone production, it should

still be sensitive to the chimeric toxin. Current animal models suggest that chimeric toxins are both specific and potent enough to be effective against at least small volume disease. Current problems in testing recombinant fusion toxins in humans include difficulty in making and purifying sufficient quantities of protein, and dealing with anti-toxin antibodies produced by some patients. Once these problems are solved and these agents move into clinical trials, we will be able to deal with issues related to chimeric toxin potency and target specificity.

REFERENCES

ALLURED, V. S., COLLIER, R. J., CARROLL, S. F. & MCKAY, D. B. (1986). Structure of exotoxin A of *Pseudomonas aeruginosa* at 3.0 Angstrom resolution. *Proc. natn. Acad. Sci. U.S.A.*, **83**, 1320–1324.

BATRA, J. K., FITZGERALD, D., GATELY, M., CHAUDHARY, V. K. & PASTAN, I. (1990). Anti-Tac(Fv)–PE40: a single chain antibody *Pseudomonas* fusion protein directed at interleukin 2 receptor bearing cells. *J. biol. Chem.*, **265**, 15198–15202.

BAUER, J., LENGYEL, G., BAUER, T. M., ACS, G. & GEROK, W. (1989). Regulation of interleukin 6 receptor expression in human monocytes and hepatocytes. *FEBS Lett.*, **249**, 27–30.

CASE, J. P., LORBERBOUM-GALSKI, H., LAFYATIS, R., FITZGERALD, D., WILDER, R. L. & PASTAN, I. (1989). Chimeric cytotoxin IL2–PE40 delays and mitigates adjuvant-induced arthritis in rats. *Proc. natn. Acad. Sci. U.S.A.*, **86**, 287–291.

CHAUDHARY, V. K., FITZGERALD, D. J., ADHYA, S. & PASTAN, I. (1987). Activity of a recombinant fusion protein between transforming growth factor type *α* and *Pseudomonas* toxin. *Proc. natn. Acad. Sci. U.S.A.*, **84**, 4538–4542.

CHAUDHARY, V. K., GALLO, M. G., FITZGERALD, D. J. & PASTAN, I. (1990a). A recombinant single-chain immunotoxin composed of anti-Tac variable regions and a truncated diphtheria toxin. *Proc. natn. Acad. Sci. U.S.A.*, **87**, 9491–9494.

CHAUDHARY, V. K., JINNO, Y., FITZGERALD, D. & PASTAN, I. (1990b). *Pseudomonas* exotoxin contains a specific sequence at the carboxyl terminus that is required for cytotoxicity. *Proc. natn. Acad. Sci. U.S.A.*, **87**, 308–312.

CHAUDHARY, V. K., JINNO, Y., GALLO, M. G., FITZGERALD, D. & PASTAN, I. (1990c). Mutagenesis of *Pseudomonas* exotoxin in identification of sequences responsible for the animal toxicity. *J. biol. Chem.*, **265**, 16306–16310.

CHAUDHARY, V. K., QUEEN, C., JUNGHANS, R. P., WALDMANN, T. A., FITZGERALD, D. J. & PASTAN, I. (1989). A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature*, **339**, 394–397.

EDWARDS, G. M., DEFEO-JONES, D., TAI, J. Y., VUOCOLO, G. A., PATRICK, D. R., HEIMBROOK, D. C. & OLIFF, A. (1989). Epidermal growth factor receptor binding is affected by structural determinants in the toxin domain of transforming growth factor-alpha-*Pseudomonas* exotoxin fusion proteins. *Molec. Cell Biol.*, **9**, 2860–2867.

FITZGERALD, D. J. P., WALDMANN, T. A., WILLINGHAM, M. C. & PASTAN, I. (1984). *Pseudomonas* exotoxin-anti-TAC. Cell-specific immunotoxin against cells expressing the human T cell growth factor receptor. *J. clin. Invest.*, **74**, 966–971.

HEIMBROOK, D. C., STIRDIVANT, S. M., AHERN, J. D., BALISHIN, N. L., PATRICK, D. R., EDWARDS, G. M., DEFEO-JONES, D., FITZGERALD, D. J., PASTAN, I. & OLIFF, A. (1990). Transforming growth factor *α*–*Pseudomonas* exotoxin fusion protein prolongs survival of nude mice bearing tumor xenografts. *Proc. natn. Acad. Sci. U.S.A.*, **87**, 4697–4701.

HENDLER, F. J. & OZANNE, B. W. (1984). Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. clin. Invest.*, **74**, 647–651.

HWANG, J., FITZGERALD, D. J., ADHYA, S. & PASTAN, I. (1987). Functional domains of *Pseudomonas* exotoxin identified by deletion analysis of the gene expressed in *E. coli*. *Cell*, **48**, 129–136.

JONES, N. R., ROSSI, M. L., GREGORIOU, M. & HUGHES, J. T. (1990). Epidermal growth factor receptor expression in 72 meningiomas. *Cancer*, **66**, 152–155.

KAWANO, M., HIRANO, T., MATSUDA, T., TAGA, T., HORII, Y., IWATO, K., ASAOKU, H., TANG, B., TANABE, O., TANAKA, H., KURAMOTO, A. & KISHIMOTO, T. (1988). Autocrine generation and requirement of BSF-2/IL-6 for human multiple myelomas. *Nature*, **332**, 83–85.

KONDO, T., FITZGERALD, D., CHAUDHARY, V. K., ADHYA, S. & PASTAN, I. (1988). Activity of immunotoxins constructed with modified *Pseudomonas* exotoxin A lacking the cell recognition domain. *J. biol. Chem.* **263**, 9470–9475.

KOZAK, R. W., LORBERBOUM-GALSKI, H., JONES, L., PURI, R. K., WILLINGHAM, M. C., MALEK, T., FITZGERALD, D. J., WALDMANN, T. A. & PASTAN, I. (1990). IL-2–PE40 prevents development of tumors in mice injected with IL-2 receptor expressing EL4 transfectant tumor cells. *J. Immun.*, **45**, 2766–2771.

KREITMAN, R. J., CHAUDHARY, V. K., WALDMANN, T., WILLINGHAM, M. C., FITZGERALD, D. J. & PASTAN, I. (1990). The recombinant immunotoxin anti-Tac(Fv) – *Pseudomonas* exotoxin 40 is cytotoxic toward peripheral blood malignant cells from patients with adult T-cell leukemia. *Proc. natn. Acad. Sci. U.S.A.*, **87**, 8291 – 8295.

LAU, J. L. T., FOWLER, J. E. JR & GHOSH, L. (1988). Epidermal growth factor in the normal and neoplastic kidney and bladder. *J. Urol.*, **139**, 170 – 175.

LORBERBOUM-GALSKI, H., BARRET, L. V., KIRKMAN, R. L., OGATA, M., WILLINGHAM, M. C., FITZGERALD, D. J. & PASTAN, I. (1989). Cardiac allograft survival in mice treated with IL-2 – PE40. *Proc. natn. Acad. Sci. U.S.A.*, **86**, 1008 – 1012.

LORBERBOUM-GALSKI, H., FITZGERALD, D., CHAUDHARY, V., ADHYA, S. & PASTAN, I. (1988a). Cytotoxic activity of an interleukin 2-*Pseudomonas* exotoxin chimeric protein produced in *Escherichia coli*. *Proc. natn. Acad. Sci. U.S.A.*, **85**, 1922 – 1926.

LORBERBOUM-GALSKI, H., GARSIA, R. J., GATELY, M., BROWN, P. S., CLARK, R. E., WALDMANN, T. A., CHAUDHARY, V. K., FITZGERALD, D. J. P. & PASTAN, I. (1990). IL2 – $PE66^{4Glu}$, a new chimeric protein cytotoxic to human activated T-lymphocytes. *J. biol. Chem.*, **265**, 16311 – 16317.

LORBERBOUM-GALSKI, H., KOZAK, R. W., WALDMANN, T. A., BAILON, P., FITZGERALD, D. J. P. & PASTAN, I. (1988b). Interleukin 2 (IL2) – PE40 is cytotoxic to cells displaying either the p55 or the p70 subunit of the IL2 receptor. *J. biol. Chem.*, **263**, 18650 – 18656.

LORBERBOUM-GALSKI, H., LAFYATIS, R., CASE, J. P., FITZGERALD, D., WILDER, R. L. & PASTAN, I. (1991). Administration of IL2 – PE40 via osmotic pumps prevents adjuvant induced arthritis in rats. Improved therapeutic index of IL-2-PE40 administered by continuous infusion. *Int. J. Immunopharmac*, **13**, 305 – 311.

OGATA, M., CHAUDHARY, V. K., FITZGERALD, D. J. & PASTAN, I. (1989). Cytotoxic activity of a recombinant fusion protein between interleukin 4 and *Pseudomonas* exotoxin. *Proc. natn. Acad. Sci. U.S.A.*, **86**, 4215 – 4219.

OGATA, M., CHAUDHARY, V. K., PASTAN, I. & FITZGERALD, D. J. (1990). Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J. biol. Chem.*, **265**, 20678 – 20685.

OGATA, M., LORBERBOUM-GALSKI, H., FITZGERALD, D. & PASTAN, I. (1988). IL2 – PE40 is cytotoxic for activated T lymphocytes expressing IL-2 receptors. *J. Immun.*, **141**, 4224 – 4228.

PAI, L. H., GALLO, M. G., FITZGERALD, D. J. & PASTAN, I. (1991). Antitumor activity of a transforming growth factor α – *Pseudomonas* exotoxin fusion protein (TGF-α – PE40). *Cancer Res.*, **51**, 2808 – 2812.

PASTAN, I. & FITZGERALD, D. (1989). *Pseudomonas* exotoxin: chimeric toxins. *J. biol. Chem*, **264**, 15157 – 15160.

PASTAN, I., WILLINGHAM, M. C. & FITZGERALD, D. J. P. (1986). Immunotoxins. *Cell*, **47**, 641 – 648.

PRIOR, T. I., HELMAN, L. J., FITZGERALD, D. J. & PASTAN, I. (1991). Cytotoxic activity of a recombinant fusion protein between insulin-like growth factor I and *Pseudomonas* exotoxin. *Cancer Res.*, **51**, 174 – 180.

REAL, F. X., RETTIG, W. J., CHESA, P. G., MELAMED, M. R., OLD, L. J. & MENDELSOHN, J. (1986). Expression of epidermal growth factor receptor in human cultured cells and tissues: relationship to cell lineage and stage of differentiation. *Cancer Res.*, **46**, 4726 – 4731.

REYNOLDS, R. K., TALAVERA, F., ROBERTS, J. A., HOPKINS, M. P. & MENON, K. M. J. (1990). Characterization of epidermal growth factor receptor in normal and neoplastic human endometrium. *Cancer*, **66**, 1967 – 1974.

ROBB, R. J., GREENE, W. C. & RUSK, C. M. (1984). Low and high affinity cellular receptors for interleukin 2: implications for the level of Tac antigen. *J. exp. Med.*, **160**, 1126 – 1146.

SCAMBIA, G., PANICI, P. B., BATTAGLIA, F., FERRANDINA, G., ALMADORI, G., PALUDETTI, G., MAURIZI, M. & MANCUSO, S. (1991). Receptors for epidermal growth factor and steroid hormones in primary laryngeal tumors. *Cancer*, **67**, 1347 – 1351.

SEETHARAM, S., CHAUDHARY, V., FITZGERALD, D. & PASTAN, I. (1991). Increased cytotoxic activity of *Pseudomonas* exotoxin and two chimeric toxins ending in KDEL. *J. biol. Chem.*, **266**, 17376 – 17381.

SIEGALL, C. B., CHAUDHARY, V. K., FITZGERALD, D. J. & PASTAN, I. (1988). Cytotoxic activity of an interleukin 6-*Pseudomonas* exotoxin fusion protein on myeloma cells. *Proc. natn. Acad. Sci. U.S.A.*, **85**, 9738 – 9742.

SIEGALL, C. B., CHAUDHARY, V. K., FITZGERALD, D. J. & PASTAN, I. (1989a). Functional analysis of domains II, Ib, and III of *Pseudomonas* exotoxin. *J. biol. Chem.*, **264**, 14256 – 14261.

SIEGALL, C. B., EPSTEIN, S., SPEIR, E., HLA, T., FOROUGH, R., MACIAG, T., FITZGERALD, D. J. & PASTAN, I. (1991a). Cytotoxic activity of chimeric proteins composed of acidic fibroblast growth factor and *Pseudomonas* exotoxin on a variety of cell types. *FASEB J.*, **5**, 2843 – 2849.

SIEGALL, C. B., FITZGERALD, D. J. & PASTAN, I. (1990a). Cytotoxicity of IL6 – PE40 and derivatives on tumor cells expressing a range of IL6 receptor levels. *J. biol. Chem.*, **265**, 16318 – 16323.

SIEGALL, C. B., KREITMAN, R. J., FITZGERALD, D. J. & PASTAN, I. (1991b). Anti-tumor effects of IL6-*Pseudomonas* exotoxin chimeric molecules against the human hepatocellular carcinoma PLC/PRF/5 in mice. *Cancer Res.*, **51**, 2831 – 2836.

SIEGALL, C. B., NORDAN, R. P., FITZGERALD, D. J. & PASTAN, I. (1990b). Cell-specific toxicity of a chimeric protein composed of interleukin 6 and *Pseudomonas* exotoxin (IL6 – PE40) on tumor cells. *Molec. Cell. Biol.*, **10**, 2443 – 2447.

SIEGALL, C. B., SCHWAB, G., NORDAN, R. P., FITZGERALD, D. J. & PASTAN, I. (1990c). Expression of the interleukin 6 receptor and interleukin 6 in prostate carcinoma cells. *Cancer Res.*, **50**, 7786–7788.

SIEGALL, C. B., XU, Y.-H., CHAUDHARY, V. K., ADHYA, S., FITZGERALD, D. & PASTAN, I. (1989b). Cytotoxic activities of a fusion protein comprised of TGF*α* and *Pseudomonas* exotoxin. *FASEB J.*, **3**, 2647–2652.

STUDIER, F. W. & MOFFATT, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Molec. Biol.*, **189**, 113–130.

TAGA, T., KAWANISHI, Y., HARDY, R. R., HIRANO, T. & KISHIMOTO, T. (1987). Receptors for B cell stimulatory factor 2. Quantitation, specificity, distribution, and regulation of their expression. *J. exp. Med.*, **166**, 967–981.

WILDING, G., VALVERIUS, E., KNABBE, C. & GELMANN, E. P. (1989). Role of transforming growth factor-*α* in human prostate cancer cell growth. *The Prostate*, **15**, 1–2.

DAB_{389}IL-2 (Denileukin Diftitox, ONTAK): Other Potential Applications

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Abstract

DAB_{389}IL-2 (denileukin diftitox, ONTAK) is an interleukin-2 receptor (IL-2R)-specific ligand fusion protein that may potentially be selective for IL-2R–expressing malignancies. The activity of DAB_{389}IL-2 in the treatment of cutaneous T-cell lymphoma has established the feasibility of utilizing such a targeted therapeutic in disseminated disease with acceptable toxicity. Data from the phase I trial suggest that the definition of activity in other cancer types, including other non-Hodgkin's lymphomas (NHL), is warranted. Three NHL patients in this study responded, two of whom had follicular lymphomas, with the third having a primary intermediate-grade B-cell NHL that was refractory to chemotherapy and stem cell transplant. This patient has remained in complete remission over 3 years after treatment with DAB_{389}IL-2. Patients treated to date have had IL-2R–positive tumors, but this remains a very complex clinical issue. The need for a threshold level of receptor expression, the difficulty in obtaining representative tissue, the lack of an assay that accurately reflects high-affinity receptor, and the potential difficulty of observer variability in evaluating the assays should point us toward examining response rates in cancer patients where IL-2R cannot be detected or is unknown. The potential to target the high-affinity IL-2R supports the development of this agent in transplantation and in autoimmune diseases. Targeting IL-2R–expressing lymphocytes may be an effective strategy for the prevention of graft rejection and to treat or prevent graft-versus-host disease. DAB_{389}IL-2 has been examined in clinical trials of psoriasis and rheumatoid arthritis and has shown promising results. The potential utility in other autoimmune disorders is unknown, but diseases such as systemic lupus, scleroderma, and vasculitis also may be effective candidates for such ligand fusion therapy.

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Introduction

The rational design of new treatment modalities in cancer therapy must emphasize agents with a novel mechanism of action that can overcome tumor resistance and are selective for the tumor cells to minimize toxicity and address the systemic nature of these diseases. DAB_{389}IL-2 (denileukin diftitox, ONTAK®) is a ligand fusion protein that potentially displays these characteristics. The activity of DAB_{389}IL-2 in the treatment of cutaneous T-cell lymphoma (CTCL) further establishes the feasibility of utilizing such a targeted therapeutic in disseminated disease, with acceptable toxicity not typical of other chemotherapeutic approaches.[1,2] The potential applications for this agent will expand as we learn more about dosing, schedule, and the clinical significance of the interleukin-2 receptor (IL-2R), as well as testing DAB_{389}IL-2 in diseases that are likely to benefit by treatment with such an agent.

Dose and Schedule

As with most new agents, the nuances of dosing and scheduling have yet to be fully explored with DAB_{389}IL-2. In contrast to some biologics, dose may be particularly important since this agent functions in a direct cytotoxic capacity in vitro and in preclinical models. In the pivotal trial in CTCL, a dose response was also seen in patients with advanced disease (Table 1). At 18 µg/kg, 38% of patients with advanced CTCL responded compared to 10% at the 9 µg/kg dose. Schedule may also assume significance with this agent. In preclinical models, cell kill was a function of both the concentration of DAB_{389}IL-2 and the exposure time. Increasing the dose from 9 µg/kg to 18 µg/kg is associated with a proportional increase in serum concentrations of DAB_{389}IL-2. Moving from a daily x 5 schedule to a less frequent administration may allow higher doses to be delivered along with a higher peak level and perhaps a better chance for response.

Assays for the Interleukin-2 Receptor

Another question arising from the trials performed to date is the relevance of the IL-2R assays. The mechanism of action is clearly mediated through the high-affinity IL-2R in vitro. The clinical problem has been to develop an assay that identifies IL-2R–expressing cells sensitive to the effects of DAB_{389}IL-2. Most effort has been focused on the development of immunofloure-sence assays for p55 and p75.[3,4] These assays are inherently limited in that they cannot predict receptor function. Further, a cell may need thousands of receptors to stain with an immunoflouresence assay, so these assays may be too insensitive to be clinically useful. Finally, cancers, especially CTCL, are frequently infiltrated with normal, activated lymphocyte popula-

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Table 1 Response in Phase III CTCL Trial

$DAB_{389}IL\text{-}2$ Dose	9 µg/kg/day		18 µg/kg/day		All	
Stage	n	Response	n	Response	n	Response
All	35	8 (23%)	36	13 (36%)	71	21 (30%)
≤ IIA	14	6 (43%)	12	4 (33%)	26	10 (38%)
≥ IIB*	21	2 (10%)	24	9 (38%)	45	11 (24%)

* Logistic regression favoring high dose for stage ≥ IIB ($P = 0.07$)
Abbreviation: CTCL = cutaneous T-cell lymphoma

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tions that may be hard to distinguish from tumor cells. Thus, the need for a threshold level of receptor expression, the difficulty in obtaining representative tissue, the lack of an assay that accurately reflects the high-affinity receptor, and the potential difficulty of observer variability in evaluating the assays should point us toward examining response rates in patients where IL-2R are negative or unknown.

Non-Hodgkin's Lymphoma

Data from the phase I trial of this interleukin-2 (IL-2) fusion protein also suggest that definition of activity in other cancer types is warranted.[1] $DAB_{389}IL\text{-}2$ has shown activity in intermediate- and low-grade non-Hodgkin's lymphomas (Table 2). One patient with an intermediate-grade NHL that was refractory to chemotherapy and stem cell transplant has remained in complete remission over 4 years after treatment with $DAB_{389}IL\text{-}2$. Phase II studies in follicular lymphomas are also planned as a result of antitumor activity in these patients. Such a trial has just opened in the Eastern Cooperative Oncology Group. Additional trials in non-Hodgkin's lymphomas will need to address the impact of histology, stage, and tumor burden on response.

Multidrug Resistance

Multiagent therapy may be particularly effective with $DAB_{389}IL\text{-}2$. Non-Hodgkin's lymphoma would be an ideal cancer to explore the addition of $DAB_{389}IL\text{-}2$ to established regimens, particularly in intermediate- and high-grade lymphomas where multiagent therapies represent a standard of care. Since it has a safety profile that complements traditional cytotoxic chemotherapy, a natural extension of studies performed to date would be to combine $DAB_{389}IL\text{-}2$ with accepted chemotherapies for these cancers.[5] There is a distinct potential for synergy here because of the unique mechanism of action of $DAB_{389}IL\text{-}2$.

By inhibiting protein synthesis, many of the strategies for repair or the development of drug resistance utilized by cancer cells might be rendered ineffective. Ligand fusion proteins with diphtheria toxin and other ribosylating toxins have been evaluated against multidrug-resistant and radiation-resistant leukemia cell lines.[6,7] These studies demonstrate that such resistant cell lines are sensitive to the effects of ligand toxins. One might further expect that agents such as $DAB_{389}IL\text{-}2$ might have a role in rendering resistant cell lines more sensitive to chemotherapy where the resistance is mediated by proteins. The feasibility of combining targeted toxins with chemotherapy has been established in clinical trials.[8] The redundant mechanisms of chemoresistance mediated by glycoproteins such as P-glycoprotein, multidrug-resistance protein, and lung-resistance protein should all be subject to the effects of protein synthesis inhibition mediated by diphtheria toxin. In addition, a p53-deficient cell line has demonstrated effective and selective cell killing, suggesting that such constructs can circumvent the failure of some malignant cells to engage in apoptotic pathways.

Transplant Medicine

An important area to explore in the development of $DAB_{389}IL\text{-}2$ is transplant medicine. In the setting of solid organ transplant, acute and chronic graft rejection mediated by host lymphocytes remains a barrier to successful treatment of some patients. In the setting of allogeneic stem cell transplantation, it is the donor lymphocytes that mediate graft-versus-host disease (GvHD), triggered by allorecognition of major and minor histocompatibility antigens. In both settings, strategies of prevention and treatment of these problems center on broadly immunosuppressive agents with their attendant complications. In either setting, once recognition has occurred, alloreactive lymphocytes express IL-2R.[9] Presumably, it is this group of IL-2R–expressing cells that mediate rejection (or GvHD in the setting of stem cell transplant).

Treatment with an IL-2R–targeted toxin affords the opportunity to selectively delete those clones involved with rejection while preserving resting lymphocytes and thus host immunity. Initial animal studies with an earlier version of $DAB_{389}IL\text{-}2$ demonstrated the abolition of the murine T-cell–dependent immune delayed type hypersensitivity.[10] Animal models of both cardiac and pancreas islet cell transplants demonstrate significant improvement in graft survival in animals treated with an IL-2 toxin.[11,12] While human studies of $DAB_{389}IL\text{-}2$ in the treatment or prevention of graft rejection or GvHD have not

Table 2 Response to $DAB_{389}IL\text{-}2$ in Phase I Clinical Trial[1]

	Cutaneous T-Cell Lymphoma	Intermediate- and Low-Grade Non-Hodgkin's Lymphoma	High-Grade Non-Hodgkin's Lymphoma
No. of Patients	35	13	4
CR	5	1	0
PR	8	2	0

begun, the feasibility of targeting the receptor for IL-2 has been established.[13] Daclizumab, a humanized monoclonal antibody to the TAC (CD25) subunit of the IL-2R complex, was recently approved by the Food and Drug Administration for use in renal transplant. This monoclonal antibody was shown to significantly reduce renal allograft rejection episodes when added to triple and double immunosuppressive protocols. Given that the *de novo* expression of the IL-2R is a critical and pivotal event in the initiation of the immune response, it is likely that such strategies would be even more effective in the setting of prophylaxis.

In allogeneic stem cell transplant, it has long been known that depleting the mature T cells from the graft could prevent GvHD. Unfortunately, T cells exert an important effect in achieving engraftment as well as against the malignancy being treated. Such T-cell–depleted grafts are therefore universally associated with a high incidence of graft failure and malignant relapse. DAB_{389}IL-2 may offer a novel approach to T-cell depletion. By mixing the allogeneic stem cell graft with irradiated host cells in order to allow those alloreactive cells to activate, DAB_{389}IL-2 in theory could deplete the graft of alloreactive IL-2R–expressing cells. This approach would presumably allow alloreactive lymphocytes to functionally declare themselves, leaving resting lymphocytes alone. Such functional T-cell depletion appears promising from some preclinical studies.[14]

Autoimmune Diseases

Although the etiology of rheumatoid arthritis (RA) has not been defined, it is a disease in which activated T-lymphocytes appear to play an important role. Since there is limited distribution of the high-affinity IL-2R beyond activated lymphocytes, IL-2R–targeted therapies should be of interest in RA. A double-blind, placebo-controlled trial of the earlier version of DAB_{389}IL-2 (DAB_{486}IL-2) that was performed in patients with refractory RA[15] demonstrated that some patients experienced clinical improvement after receiving one or more 5-day courses of DAB_{486}IL-2. The results of further trials to characterize the activity of DAB_{389}IL-2 in these and other patients with RA are pending.

Other autoimmune diseases in which the use of DAB_{389}IL-2 could be considered would be systemic lupus, inflammatory bowel disease, and psoriasis. The latter is supported by existing preclinical data.[16] In these studies, DAB_{389}IL-2 selectively targeted and eliminated activated IL-2R–positive lymphocytes from the lamina propria. Psoriasis also appears to be a systemic autoimmune disease that manifests primarily in the skin. As in RA, activated T-lymphocytes are thought to exert a central role in the pathogenesis of this disease. One phase II multicenter trial that looked at DAB_{389}IL-2 in patients with recalcitrant psoriasis showed some benefit with low doses.[17]

HIV

Induction of cytokines and cytokine receptors, including the IL-2R, is important in the establishment of infection with HIV-1, enhancement of viral replication, and activation of HIV expression in latently infected cells.[18,19] Both DAB_{486}IL-2 and its analogue, DAB_{389}IL-2, have been evaluated for their ability to selectively eliminate HIV-infected cells in vitro before virus is produced. The rationale for this approach is based upon the observation that integration of virus into the host genome and its subsequent replication require cell activation. Because the development of high-affinity IL-2R accompanies lymphocyte activation, these infected cells represent potential targets for the cytotoxic action of an IL-2 fusion toxin. In vitro studies in HIV-infected lymphocytes and monocytes indicate that DAB_{486}IL-2 eliminates the production of HIV-1 proteins (gp160, p55, and p24 antigen) and infectious virus in mixed T-cell cultures and inhibits replication in monocytes. The IL-2 toxin does not affect the viability of uninfected, resting T cells.[18,19] Both agents were also evaluated in peripheral blood cell cultures infected with laboratory strains of HIV-1, clinical isolates of HIV-1, and zidovudine (AZT)-resistant strains of the virus.[17] These studies utilized a sensitive RNA:RNA hybridization assay to detect viral replication and showed that both IL-2 fusion toxins significantly reduced HIV-specific RNA production. Concentrations of DAB_{389}IL-2 as low as 10^{-11} mol eliminated HIV from the culture.

Conclusion

The clinical development of DAB_{389}IL-2, beginning with its predecessor, DAB_{486}IL-2, looked at many different disease entities before focusing on CTCL. Activity was seen in intermediate- and low-grade non-Hodgkin's lymphomas as a single agent. Its mechanism of action suggests that combination therapies with DAB_{389}IL-2 may have a synergistic effect in certain tumors. As a targeted therapy, DAB_{389}IL-2 may play a role in selectively deleting the T-cell clones that ultimately lead to GvHD and rejection in stem cell transplant. For the same reasons, DAB_{389}IL-2 may selectively eliminate HIV-infected cells. Finally, there may well be a role for DAB_{389}IL-2 in autoimmune diseases such as RA, where activated T-lymphocytes can be targeted. Now that DAB_{389}IL-2 has been approved for CTCL, it is hoped that investigators will develop studies to explore some of these other potential applications for DAB_{389}IL-2.

References

1. LeMaistre CF, Saleh MN, Kuzel TM, et al. Phase I trial of a ligand fusion-protein (DAB_{389}IL-2) in lymphomas expressing the receptor for interleukin-2. *Blood* 1998; 91:399-405.
2. Kuzel T, Olsen E, Martin A, et al. Pivotal phase III trial of two dose levels of DAB_{389}IL-2 (Ontak™) for the treatment of mycosis fungoides (MF). *Blood* 1997; 90:586a (Abstract #2607).
3. LeMaistre CF, Craig FE, Meneghetti C, et al. Phase I trial of a 90-minute infusion of the fusion toxin DAB_{486}IL-2 in hematological cancers. *Cancer Res* 1993; 53:3930-3934.
4. Foss FM, Borkowski TA, Gilliom M, et al. Chimeric fusion protein toxin DAB_{486}IL-2 in advanced mycosis fungoides and the Sezary syndrome: correlation of activity and interleukin-2 receptor expression in a phase II study. *Blood* 1994; 84:1765-1774.
5. Fisher RI, Gaynor ER, Dahlberg S, et al. A phase III comparison of CHOP vs. m-BACOD vs. ProMACE-CytaBOM vs. MACOP-B in patients with intermediate- or high-grade non-Hodgkin's lymphoma: results of SWOG-8516 (Intergroup 0067), the National High-Priority Lymphoma Study. *Ann Oncol* 1994; 5:91-95.
6. Perentesis JP, Waddick KG, Bendel AE, et al. Induction of apoptosis in multidrug-resistant and radiation-resistant acute myeloid leukemia

cells by a recombinant fusion toxin directed against the human granulocyte macrophage colony-stimulating factor receptor. *Clin Cancer Res* 1997; 3:347-355.
7. O'Connor R, Liu C, Ferris CA, et al. Anti-B4-blocked ricin synergizes with doxorubicin and etoposide on multidrug-resistant and drug-sensitive tumors. *Blood* 1995; 86:4286-4294.
8. Scadden DT, Schenkein DP, Bernstein Z, et al. Immunotoxin combined with chemotherapy for patients with AIDS-related non-Hodgkin's lymphoma. *Cancer* 1998; 83:2580-2587.
9. Cantrell DA, Smith KA. The interleukin-2 T-cell system: a new cell growth model. *Science* 1984; 224:1312-1316.
10. Kelley VE, Bacha P, Pankewycz O, et al. Interleukin 2-diphtheria toxin fusion protein can abolish cell-mediated immunity in vivo. *Proc Natl Acad Sci USA* 1988; 85:3980-3984.
11. Pankewycz O, Mackie J, Hassarjian R, et al. Interleukin-2-diphtheria toxin fusion protein prolongs murine islet cell engraftment. *Transplantation* 1989; 47:318-322.
12. Kirkman RL, Bacha P, Barrett LV, et al. Prolongation of cardiac allograft survival in murine recipients treated with a diphtheria toxin-related interleukin-2 fusion protein. *Transplantation* 1989; 47:327-330.
13. Vincenti F, Kirkman R, Light S, et al. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. *N Engl J Med* 1998; 338:161-165.
14. Harris DT, Sakiestewa D, Lyons C, et al. Prevention of graft-versus-host disease (GVHD) by elimination of recipient-reactive donor T cells with recombinant toxins that target the interleukin 2 (IL-2) receptor. *Bone Marrow Transplant* 1999; 23:137-144.
15. Moreland LW, Sewell KL, Trentham DE, et al. Interleukin-2 diphtheria fusion protein ($DAB_{486}IL\text{-}2$) in refractory rheumatoid arthritis. A double-blind, placebo-controlled trial with open-label extension. *Arthritis Rheum* 1995; 38:1177-1186.
16. Bousvaros A, Stevens AC, Strom TB, et al. Interleukin-2 fusion protein ($DAB_{389}IL\text{-}2$) selectively targets activated human peripheral blood and lamina propria lymphocytes. *Dig Dis Sci* 1997; 42:1542-1548.
17. Bagel J, Garland WT, Breneman D, et al. Administration of $DAB_{389}IL\text{-}2$ to patients with recalcitrant psoriasis: a double-blind, phase II multicenter trial. *J Am Acad Dermatol* 1998; 38:938-944.
18. Finberg RW, Wahl SM, Allen JB, et al. Selective elimination of HIV-1-infected cells with an interleukin-2 receptor-specific cytotoxin. *Science* 1991; 252:1703-1705.
19. Zhang L, Waters C, Nichols J, et al. Inhibition of HIV-1 RNA production by the diphtheria toxin-related IL-2 fusion proteins $DAB_{486}IL\text{-}2$ and $DAB_{389}IL\text{-}2$. *J Acquir Immune Defic Syndr* 1992; 5:1181-1187.

Interleukin-2 Fusion Toxin: Targeted Therapy for Cutaneous T Cell Lymphoma

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ABSTRACT: **The interleukin (IL)-2 receptor has proved an attractive target for T cell–directed therapies. Agents including monoclonal antibodies, single-chain antibody immunoconjugates, radioimmunoconjugates, and, most recently, ligand fusion toxins have demonstrated activity *in vitro* and in clinical trials in both hematologic malignancies and diseases characterized by proliferation of activated T cells, such as graft-versus-host disease. DAB_{389}IL-2 (ONTAK) is a ligand fusion toxin consisting of the full-length sequence of the IL-2 gene genetically fused to the enzymatically active and translocating domains of diphtheria toxin. DAB_{389}IL-2 and its predecessor, DAB_{486}IL-2, have demonstrated activity in a variety of diseases, including cutaneous T cell lymphoma (CTCL), psoriasis, rheumatoid arthritis, and HIV infection. Further clinical development of IL-2 fusion toxins in CTCL and other hematopoietic malignancies is predicated on identification of the high-affinity IL-2 receptor complex on the malignant cells and on a better understanding of the biological determinants of cytotoxicity of these molecules *in vivo*.**

KEYWORDS: **interleukin-2 fusion toxins; T cell–directed therapies; diphtheria toxin**

INTRODUCTION

The diphtheria toxin (DT) ligand fusion proteins are a class of novel agents that, like immunotoxins, are capable of delivering a cytotoxin into the intracellular compartment. In the case of immunotoxins, a plant or bacterial toxin is chemically conjugated to either a complete or a single-chain antibody. Fusion toxins represent recombinant chimeric proteins in which the sequence for the toxin moiety is genetically fused to the targeting ligand.

Both plant and animal toxins exert their cytotoxic activity by interfering with cellular protein synthesis machinery. Native DT is a 535–amino acid protein consisting of three domains: an enzymatically active domain (fragment A); a hydrophobic domain (N terminal portion of fragment B); and the receptor binding domain (C terminal portion of fragment B).[1,2] DT intoxicates sensitive eukaryotic cells by binding to cell surface receptors, whereupon it is internalized into endosomal vesicles by receptor-mediated endocytosis. Following acidification of the endocytic vesicle, cleavage of the catalytically active domain of DT occurs, thus liberating it into the

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cytosol, where it ADP-ribosylates elongation factor 2, thus leading to inhibition of protein synthesis.[3–7] *In vitro*, a single molecule of DT is capable of intoxicating a sensitive cell.

DAB_{486}IL-2 was the first recombinant ligand toxin fusion protein tested both *in vitro* and in animals. DAB_{486}IL-2 encodes a 60-kDa protein, which contains the first 486 amino acids of DT and the full-length sequence for IL-2.[8,9] The recombinant fusion protein was demonstrated to be biologically active as a ligand in that it was capable of binding and initiating signal transduction events in cells expressing IL-2 receptor (IL-2R).[10] Subsequently, because of the short half-life and unfavorable pharmacodynamics of DAB_{486}IL-2, successive truncations of the DT portion of the molecule were undertaken, and bioactivity was optimized when only the first 389 amino acids of DT were used in the fusion protein. This molecule, DAB_{389}IL-2 (ONTAK), was shown to have a two- to threefold higher molecular mass than DAB_{486}IL-2, with a 10-fold increase in potency.[11]

The susceptibility of both normal and neoplastic cells to cytotoxicity from the IL-2 fusion toxins is dependent on the expression of the high-affinity IL-2R, consisting of the p55 (α), p75 (β), and p64 (γ) subunits.[12–15] The p55 subunit consists of a large extracellular and a small intractyoplasmic domain and is responsible for anchoring the high-affinity complex. The intracytoplasmic domain of the p75 component is critical in signal transduction and binds to the JAK kinases and other signaling molecules,[16,17] The p64 component is necessary for ligand internalization and is a constituent of a number of cytokine receptors, including IL-4, IL-7, and IL-15.[18–20] IL-2 engagement is capable of coupling either p55 or p55 p75 with p64, thereby mediating signal transduction or ligand internalization. Intermediate-affinity IL-2R consists of p75 p64 without p55, and low-affinity receptors consist of either p55 or p75 with or without p64.

IL-2R expression, as defined by immunohistochemistry using the anti-TAC antibody, has been reported on hematopoietic malignancies, including Hodgkin's disease, low- and intermediate-grade non-Hodgkin's lymphoma, cutaneous T cell lymphoma (CTCL), HTLV-1–associated adult T cell leukemia/lymphoma, and chronic lymphocytic leukemia.[21–27] While expression of the p55 component of the receptor has been demonstrated using the anti-TAC antibody, there is little data regarding the expression of the high-affinity IL-2R on human leukemia and lymphoma cells. High-affinity receptor has been demonstrated on HTLV-1–infected T cells as well as on activated proliferating T lymphocytes, recently activated B cells, and activated monocytes. Expression of the intermediate affinity receptor has been shown on a subset of hematopoietic malignancies, including natural killer (NK) lymphomas, as well as on normal natural killer cells.[15,27] DAB_{389}IL-2 *in vitro* has been shown to intoxicate cell lines bearing the high-affinity IL-2R isoform with an IC_{50} of 10–12 M, whereas cells lacking the full complement of high-affinity receptor (p55 without p75) were relatively resistant (IC_{50} of 10^{-8} M).[11,28] Cell lines expressing intermediate-affinity receptors (p75 p64) were intoxicated with an IC_{50} of 10^{-10} M. It has been demonstrated that only a small number of high-affinity receptors are required on the cell for intoxication. The minimum contact time for internalization is 15–30 minutes. After binding of the fusion toxin, an initial signaling event is observed *in vitro*, mimicking that of IL-2, in which mRNA expression for IL-2, IL-2R, *c*-myc, and interferon-γ increase.[10] Protein synthesis is inhibited within 4–6 hours, with apoptosis ensuing within 40–72 hours.

INITIAL CLINICAL TRIALS WITH INTERLEUKIN-2 FUSION TOXINS

The initial clinical trials with DAB_{486}IL-2 were designed as cohort dose escalations in which single and multiple doses of DAB_{486}IL-2 were administered by intravenous injection either as a bolus or as a 90-minute infusion in patients with hematologic malignancies.[28–33] In these trials, IL-2R expression was measured but was not a prerequisite for entry. At doses ranging from 700 ng/kg/d to 400 μg/kg/d, the most frequently occurring adverse events in these studies included hypersensitivity, fever, malaise, chills, and transient elevation of serum hepatic transaminases. The maximal tolerated dose for DAB_{486}IL-2 was 400 μg/kg/d, above which renal insufficiency occurred.[29] The half-life of DAB_{486}IL-2 was approximately 11 minutes at dose levels of 200–400 μg/kg. While a third of patients had significant preexisting titers of anti-DT antibodies from childhood immunizations, over 60% boosted their titers during the study, and more than 80% had measurable titers after DAB_{486}IL-2 administration. The presence of preexisting anti-DT antibodies did not preclude response to therapy. Few patients, however, had anti-IL-2 titers prior to study entry.

Clinical responses were documented in patients with low- and intermediate-grade B cell non-Hodgkin's lymphoma, Hodgkin's disease, and CTCL. All of these patients had refractory disease and had failed at least two prior chemotherapy regimens. While IL-2R expression on the patient's tumor tissue was not a requirement for entry onto this study, immunohistochemical studies were performed in patients with tissue readily accessible for biopsy. All of the responders had demonstrable IL-2R expression as measured by immunoreactivity with the anti-TAC antibody. The most impressive responses to DAB_{486}IL-2 were noted in patients with CTCL, in whom the response rate was 17% (TABLE 1). One patient with tumor-stage CTCL had a complete remission, with disappearance of all of his tumors, and he has remained disease free without further therapy for at least eight years.[31] There were four responses in patients with B cell non-Hodgkin's lymphoma, including a complete response (CR), lasting over 18 months, in a patient with an intermediate-grade follicular large-cell lymphoma. Partial responses (PRs) were seen in two additional patients, one with follicular small-cell lymphoma and one with CLL, with remissions lasting 12 and 6 months, respectively. A minor response was observed in a patient with diffuse small cleaved lymphoma.

A phase II study of DAB_{486}IL-2 was performed in patients with CTCL.[34] In this study, which used a 5-day dosing regimen every 21 days, 15 patients with refractory CTCL were enrolled. One patient with diffuse plaque–stage disease had a PR, with significant clearing of his lesions; and two patients with the Sézary syndrome experienced marked improvement in skin exfoliation and pruritis without a significant

TABLE 1. Clinical response to IL-2 fusion toxins in CTCL patients

Diagnosis	No. enrolled	CR	PR	Overall	Response duration
DAB_{486}IL-2	36	1	5	17%	ND
Phase I/II DAB_{389}IL-2	35	5	8	37%	3.6 months
Phase III DAB_{389}IL-2	73	7	14	30%	4.4 months

change in the numbers of circulating Sézary cells. In this study, all patients expressed a component of the IL-2R (either p55, p75, or both) on their tumor cells as measured by immunohistochemistry and reverse-transcriptase polymerase chain reaction (RT-PCR), but there was no correlation between receptor isoform and clinical response.

A phase I/II study of the truncated form of the fusion toxin, DAB_{389}IL-2 (ONTAK), was performed in patients with non-Hodgkin's lymphoma, Hodgkin's disease, and CTCL whose tumors expressed the IL-2R, as determined by immunohistochemical staining using the anti-CD25 antibody and antibodies directed against the p75 subunit of the IL-2R.[35,36] In this study, immunostaining of >25% of the patient's tumor cells by immunostaining of skin or lymph node tissue was required for enrollment. Of 73 patients enrolled in this study, 25 had undergone prior bone marrow transplantation for lymphoma. The median age was 51, and the median number of prior therapies was five.

Toxicities were similar to those observed with DAB_{486}IL-2 and included infusion-related fever and chills in 74%, and nausea/vomiting, asthenia, and mild hypotension in 50%.[35] Most of the adverse events occurred in the first two cycles of therapy, and the severity of these adverse events diminished with subsequent courses. Transient hypoalbuminemia (80%) and transient hepatic transaminase elevation (62%) were the most commonly observed laboratory abnormalities and were reversible in all patients. Of interest was a combination of hypoalbuminemia, hypotension, and edema, which was observed in eight patients with CTCL. This triad was felt to represent a mild vascular leak type of syndrome similar to that observed with ricin A chain or *Pseudomonas* exotoxin A immunotoxin molecules. The maximum tolerated dose, 27 μg/kg/d × 5 days, was defined based on the occurrence of severe fatigue and asthenia at the 31-μg/kg/d dose.

Clinical responses were observed in 13 of 35 (37%) CTCL patients and 3 of 17 (18%) B cell non-Hodgkin's lymphoma patients, including two with follicular lymphoma and one with diffuse large-cell lymphoma, who relapsed less than 100 days after autologous transplantation. The median time to response was 2 months (two cycles of treatment), with a median response duration of 10 months. No responses were noted in patients with Hodgkin's disease. The most dramatic responses, including five of the six complete responses, were observed in patients with CTCL (TABLE 1).[36] The median response duration in this group of patients was 15 months for the complete responders. Responses were noted in 2/4 patients who had previously received DAB_{486}IL-2; a third patient had disease stabilization.

Similar to prior studies with DAB_{486}IL-2, there was no dose-response relationship in this phase I/II study and no correlation between levels of neutralizing anti-DT antibody and clinical response. In addition, there was no correlation between the levels of antibody and the detection of circulating DAB_{389}IL-2, indicating that not all immunoreactive antibody was neutralizing. Pharmacokinetic studies indicated that the half-life of DAB_{389}IL-2 was 72 minutes, with superimposible clearance profiles on days 1 and 5, indicating no accumulation with repeated dosing.[35] Levels of soluble IL-2R (sIL-2R) were high in most patients prior to treatment and decreased in CTCL patients who had a clinical response. Interestingly, there was no significant change in sIL-2R in the NHL responders.

Despite the possibility that DAB_{389}IL-2 could conceivably target circulating populations of activated T cells or NK cells, there was no overall change in lymphocyte

populations coexpressing CD3, CD4, or CD8 and CD25 during therapy. Individual immunophenotypic profiles did demonstrate, however, a transient increase in CD25-expressing lymphocytes during the five days of dosing, which subsequently normalized within a few days of the last dose. There was no increased incidence of sepsis or opportunistic infections in these patients.

PHASE III STUDY OF ONTAK IN CTCL

A phase III clinical trial with $DAB_{389}IL\text{-}2$ enrolled 73 patients with advanced or refractory CTCL who were stratified by stage (I-IIB vs. IIB-IV) and were randomized to receive either 9 or 18 μg/kg/d for 5 consecutive days for 21-day cycles.[37,38] IL-2R expression was determined by immunohistochemistry on skin biopsies. Criteria for study entry was expression of CD25 on at least 20% of tumor cells in the biopsy. All patients were heavily pretreated, with a median of five prior therapies. The median time from diagnosis of CTCL until study entry was five years (range 0.3–20). The majority of patients had failed topical chemotherapy or cutaneous phototherapy, 47% had failed multiagent chemotherapy, and 51% had failed interferon.

The overall response rate was 30%, similar to the phase I study, with CRs occurring at both dose levels. The response rate was slightly higher at the higher dose (36% vs. 23%), but this was not statistically significant, nor was there a difference between the two doses with respect to number of complete and partial responders. As shown in TABLE 2, responses were noted in both early- and advanced-stage patients, including 10 patients with plaque disease (stage Ib-IIa), 6 with cutaneous tumors (IIb), 2 with erythroderma, and 3 with stage IVA disease. Responses occurred rapidly, within the first three cycles of therapy in the majority of patients. While the overall objective response rate was 30%, improvement in overall disease burden, as measured by a weighted disease assessment, including skin score and measurement of involved lymph nodes, was noted in 60% of patients. The response duration, calculated from the time of first response, was 4.4 months.

The predominant toxicities included infusion-related hypersensitivity events in up to 74% of patients, including fever, rash, hypotension, back tightness, chest tightness, shortness of breath, and myalgias. Prohibition of steroid premedication in this study likely contributed to the overall incidence of hypersensitivity events. In subsequent studies using steroid premedication, the incidence of hypersensitivity events has been substantially decreased.[39] Vascular leak syndrome occurred in 27% of patients and was manifest on days 6–10 of the cycle. No correlation was seen between

TABLE 2. Response to $DAB_{389}IL\text{-}2$ by stage in CTCL patients

	Phase I/II study No./total	Phase III study No./total
Stage I-IIa	7/12	10/26
Stage IIb	4/6	6/19
Stage III	2/7	2/11
Stage IVa	0/9	3/15

development of vascular leak syndrome and levels of anti-DT antibody, drug dose, or incidence of acute hypersensitivity. Unlike the vascular leak seen with other immunotoxin-based therapies, vascular leak associated with DAB_{389}IL-2 was relatively mild in nature. Most patients were managed without intervention or with judicious fluid replacement and diuresis.

Other toxicities included a maculopapular skin rash in up to 42% of patients. Biopsy in a number of patients was consistent with a drug eruption. Most patients were treated symptomatically and the rash resolved. Only five patients discontinued therapy due to skin rash. Reversible hepatic transaminitis occurred in over 70% of patients and resolved after the first course of therapy in the majority. Similar to the phase I study, there was no correlation between boost in titer of anti-DT antibodies and clinical response or onset of response.

In summary, both the phase I/II and phase III studies of DAB_{389}IL-2 have demonstrated activity across a range of doses in patients with CTCL. Response durations are similar (3.6–4.4 months), as are the incidence of adverse events. Responses have occurred even in patients with advanced skin stage. Because of the design of these trials, few patients with visceral involvement were enrolled, so the response rate in these patients is unknown. Since both clinical trials used the five-consecutive-day dosing schedule, it is unclear whether alternative schedules might demonstrate activity.

CORRELATION BETWEEN IL-2R ISOFORM AND CLINICAL RESPONSE

DAB_{389}IL-2 is currently approved for the treatment of patients with CTCL whose tumor cells express IL-2 receptor, as defined by immunohistochemical staining using antibodies (CD25) directed at the p55 component of the receptor. Currently, the immunoassay for CD25 expression must be performed on frozen tissue, although paraffin-based assays are under development. The sensitivity and predictive value of the CD25 assay have not been well defined, since only a few patients who were CD25 negative by immunohistochemistry have been treated with IL-2 fusion toxins.

We performed a retrospective analysis of the IL-2R isoform, as defined by immunohistochemical detection of the p55 and p75 receptor components, in 129 patients screened for entry into the phase I/II DAB_{389}IL-2 study.[40] As shown in TABLE 3, all 15 patients with Hodgkin's disease expressed a component of the receptor, with 7 expressing p55/p75 and 8 expressing p55 alone. Of 58 CTCL patients screened, 33 expressed p55, 19 expressed p55/p75, and 6 expressed p75 alone. Of patients with low-grade B cell NHL, 14 of 31 expressed p55, and none expressed p75. Of 35 with intermediate-grade NHL, 10 expressed p55, and 5 expressed p55/p75. When the IL-2R isoform was correlated with clinical response, as shown in TABLE 4, there was no difference in response rates between p55 and p55/p75 for either the CTCL patients or the patients with intermediate-grade B cell NHL. One patient with low-grade NHL with low-affinity receptor (p55) and one with CTCL, who expressed intermediate-affinity receptor (p75), responded. While p64 was not measured in this study, it has been demonstrated to be ubiquitously expressed in hematopoietic neoplasms. Of the responders, all but one expressed p55, for a predictive value of 93% for CD25 expression.

TABLE 3. Expression of IL-2R by immunohistochemistry in patients with CTCL, Hodgkin's disease, and non-Hodgkin's lymphoma

Histology	No.	p55	p75	p55/75
Hodgkin's disease				
Nodular sclerosis HD	13	8		5
Lymphocyte depleted /LPHD	2			2
Non-Hodgkin's lymphoma				
Low-grade NHL	31	14	0	0
Intermediate-grade NHL	29	5	0	4
Immunoblastic lymphoma	6	5	0	1
Cutaneous T cell lymphoma	**58**	**33**	**6**	**19**

ABBREVIATIONS: HD = Hodgkin's disease; LPHD = lymphocyte-predominant Hodgkin's disease; NHL = non-Hodgkin's lymphoma.

TABLE 4. Correlation between immunohistochemical expression of IL-2R subunits and clinical response to DAB_{389}IL-2

Histology	p55	p75	p55/75
CTCL (n = 13)	8/33	1/6	4/19
NHL low grade (n =1)	1/14		
NHL intermediate grade (n =2)	1/5		1/4

ABBREVIATIONS: CTCL = cutaneous T cell lymphoma; NHL = non-Hodgkin's lymphoma.

To address the correlation between degree of immunopositivity for CD25 and clinical response, we analyzed the immunohistochemical results for the 72 CTCL patients treated on the phase III study. Immunohistochemical results were reported by a reference pathologist, who classified the slides based on CD25 immunostaining of 20–50%, or >50% of the tumor cells in the skin biopsy. Of 57 patients with 20–50% $CD25^+$ cells, 17 (30%) responded, while of the 15 with >50% $CD25^+$ cells, 4 (28%) responded, suggesting that there was no correlation between the intensity of immunostaining for CD25 and clinical response (TABLE 5). In this study, there were no low expressors (<20% $CD25^+$ cells staining) treated, so the response rate in those patients is unknown. To further define the correlation between receptor isoform and response,

TABLE 5. Correlation between response to DAB_{389}IL-2 and CD25 expression in cutaneous T cell lymphoma in phase III study

Percent of $CD25^+$ tumor cells	No. of patients	Response no. (%)
20–50%	57	17 (30%)
>50%	15	4 (28%)

a retrospective analysis of p55, p75, and p64 expression by semiquantitative RT-PCR is underway. In addition, clinical trials have been initiated to explore the clinical benefit of DAB_{389}IL-2 in CTCL and B cell NHL patients whose tumor cells lack detectable IL-2R expression using the conventional immunohistochemical assay.

MODULATION OF IL-2R EXPRESSION

IL-2R expression has been shown to be modulated *in vivo* by a number of factors, including cytokine stimulation. The p55 subunit, which is absent from resting T lymphocytes, is induced by T cell activation, viral gene products such as HTLV-1–associated TAX, protein kinase A (PKA), IL-2, IL-1, and TNF-α.[41] Likewise, the p75 subunit, which is expressed on resting T lymphocytes, is upregulated by activation, IL-2, and IL-4. The p64, or common γ subunit, is constitutively expressed but upregulated by IL-2 and IFN-γ. Recent studies by our group and others have demonstrated that both butyrates and retinoids are capable of modulating IL-2R expression.[42,43] The RAR-binding retinoid, all-trans retinoic acid (ATRA), increases p55 and p75 expression in human T cell leukemia cell lines. We recently demonstrated similar results for the RXR-binding retinoid bexarotene. To demonstrate that these retinoids are capable of upregulating expression of the high-affinity IL-2R, we exposed human leukemia cells to retinoids, measured expression of the IL-2R subunits by Western blot and by RT-PCR, and then determined susceptibility of the cells to DAB_{389}IL-2. We reported that both the RAR- and RXR-binding retinoids are capable of upregulating both the p55 and p75 components of the receptor and enhance the sensitivity of the treated cells to intoxication by DAB_{389}IL-2. These results have led to the development of a clinical trial to explore the use of retinoids as biomodulatory agents in patients with hematologic malignancies whose tumor cells demonstrate low or absent expression of IL-2R. In similar studies, we have reported that butyrate derivatives are capable of modulating IL-2R expression as well as susceptibility of leukemia cells to intoxication by DAB_{389}IL-2. Alternative mechanisms to explain the enhanced sensitivity of the cells are being explored, including the direct effects of butyrates and other short-chain fatty acids on membrane internalization and processing of the fusion toxins.

SUMMARY: MECHANISM OF ACTION OF IL-2 FUSION TOXINS IN CTCL

DAB_{389}IL-2 as a single agent has demonstrated significant clinical activity in CTCL. Thus far, there are no predictors of response other than detectable CD25 expression, and there have been no data regarding mechanisms of resistance or lack of response in patients whose tumors have been demonstrated to express the high-affinity IL-2R. Suboptimal clinical response might be related to total drug exposure, and alternative dosing schedules are being pursued. While the purported mechanism of action of DAB_{389}IL-2 in CTCL relates to direct targeting and intoxication of IL-2R–expressing tumor cells, other possible biological activities of the agent might contribute to its observed clinical activity. One might be the modulation of normal activated T and NK cell responses in the skin and other target organs, thus disturbing

TABLE 6. Enhanced cytotoxicity to $DAB_{389}IL\text{-}2$ in HUT78 T cell lymphoma cells after exposure to retinoids or butyrate

Treatment	Percent Survival
No treatment	100
$DAB_{389}IL\text{-}2\ 10^{-12}M$	110
$DAB_{389}IL\text{-}2\ 10^{-12}M$ + butyrate 10^{-6} M	85
$DAB_{389}IL\text{-}2\ 10^{-12}M$ + butyrate 10^{-5} M	50
$DAB_{389}IL\text{-}2\ 10^{-12}M$ + ATRA 10^{-9} M	68
$DAB_{389}IL\text{-}2\ 10^{-12}M$ + ATRA 10^{-8} M	44
$DAB_{389}IL\text{-}2\ 10^{-12}M$ + butyrate 10^{-6} M + ATRA 10^{-9} M	68
$DAB_{389}IL\text{-}2\ 10^{-12}M$ + butyrate 10^{-5} M + ATRA 10^{-8} M	38

NOTE: HUT78 cells were exposed to either butyrate or all-trans retinoic acid (ATRA) for 24 hours prior to the addition of $DAB_{389}IL\text{-}2$. Cytotoxicity was measured 72 hours later by MTT assay and compared to control, untreated cells.

paracrine growth factors within the microenvironment. Another mechanism might relate to the signal transduction events resulting from ligand-receptor engagement. Potentially, receptor engagement without internalization might lead to altered cytokine secretion or possibly sensitize the cells to undergo programmed cell death. The latter mechanism might be exploited in combination therapies using $DAB_{389}IL\text{-}2$ with other cytotoxic agents.

REFERENCES

1. GREENFIELD, L., V. JOHNSON & R. YOULE. 1987. Mutations in diphtheria toxin separate binding from entry and amplify immunotoxin selectivity. Science **238:** 536–539.
2. ROLF, J. & L. EIDELS. 1993. Structure function analyses of diphtheria toxin by use of monoclonal antibodies. Infect. Immunol. **61:** 944–1003.
3. MOYA, M., A. DAUTRY-VERSAT, B. GOUD, *et al.* 1985. Inhibition of coated pit formation in Hep_2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. J. Cell Biol. **101:** 548–559.
4. MORRIS, R.E., A.S. GERSTEIN, P.F. BONVENTRE & C.B. SAELINGER. 1985. Receptor-mediated entry of diphtheria toxin into monkey kidney (Vero) cells: electron microscopic evaluation. Infect. Immunol. **50:** 721–727.
5. FUCHS, R., S. SCHMID & I. MELLMAN. 1989. A possible role for Na^+, K^+-ATPase in regulating ATP-dependent endosome acidification. Proc. Natl. Acad. Sci. USA **86:** 539–543.
6. KAGAN, B.L., A. FINKELSTEIN & M. COLOMBINI. 1981. Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. Proc. Natl. Acad. Sci. USA **78:** 4950–4954.
7. DONOVAN, J.J., M.I. SIMON, R.K. DRAPER *et al.* 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. Proc. Natl. Acad. Sci. USA **78:** 172–176.
8. WILLIAMS, D., K. PARKER, W. BISHAI, *et al.* 1987. Diphtheria-toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. Protein Eng. **1:** 493–498.
9. BACHA, P., C. WATERS, J. WILLIAMS, *et al.* 1988. Interleukin-2 targeted cytotoxicity: selective action of a diphtheria toxin-related interleukin-2 fusion toxin. J. Exp. Med. **167:** 612–622.

10. WALZ, G,, B. ZANKER, K. BRAND, *et al.* 1989. Sequential effects of interleukin-2 diphtheria toxin fusion protein on T-cell activation. Proc. Natl. Acad. Sci. USA **86:** 9485–9488.
11. KIYOKAWA, T., D. WILLIAMS, C. SNIDER, *et al.* 1991. Protein engineering of diphtheria toxin-related interleukin-2 fusion toxins to increase cytotoxic potency for high affinity IL-2 receptor bearing target cells. Protein Eng. **4:** 463–468.
12. LEONARD, W., J. DEPPER, G. CRABTREE, *et al.* 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. Nature **311:** 626–631.
13. TSUDO, M., R. KOZAK, C. GOLDMAN, *et al.* 1986. Demonstration of a non-TAC peptide that binds interleukin 2: a potential participant in a multichain interleukin-2 receptor complex. Proc. Natl. Acad. Sci.USA **83:** 9694–9698.
14. TAKESHITA, T., H. ASAO, K. OHTANI, *et al.* 1992. Cloning of the γ chain of the human IL-2 receptor. Science **257:** 379–382.
15. WALDMANN, T.A. 1990. The multichain interleukin-2 receptor. A target for immunotherapy in lymphoma, autoimmune disorders, and organ allografts. JAMA **263:** 272–274.
16. FARRAR, W. & D. FERRIS. 1989. Two-dimensional analysis of interleukin 2-regulated tyrosine kinase activation mediated by p75 β subunit of the interleukin-2 receptor. J. Biol. Chem. **264:** 12562–12567.
17. Merida, I. & G. Gaulton. 1990. Protein tyrosine phosphorylation associated with activation of the interleukin-2 receptor. J. Biol. Chem. **265:** 5690–5694.
18. KONDO, M., T. TAKESHITA, N. ISHII, *et al.* 1993. Sharing of the interleukin-2 receptor γ chain between receptors for IL-2 and IL-4. Science **62:** 1874–1877.
19. KONDO, M., T. TAKESHITA & M. HIGUCHI. 1994. Functional participation of the IL-2 Rγ chain in IL-7 receptor complexes. Science **263:** 1453–1454.
20. GIRI, J., M. AHDIEH, J. EISENMAN, *et al.* 1994. Utilization of the β and γ chains of the IL-2 R by the novel cytokine IL-15. EMBO J. **13:** 2822–2830.
21. WALDMANN, T.A. 1986. The structure, function, and expression of interleukin-2 receptors on normal and malignant T cells. Science **232:** 727–732.
22. STRAUCHEN, J. & B. BREAKSTONE. 1987. IL-2 receptor expression in human lymphoid lesions. Am. J. Pathol. **126:** 506–512.
23. SHEIBANI, K., C. WINBERG, S. VAN DE VELDE, *et al.* 1987. Distribution of lymphocytes with interleukin-2 receptors (TAC antigens) in reactive lymphoproliferative processes, Hodgkin's disease, and non-Hodgkin's lymphomas. An immunohistochemical study of 300 cases. Am. J. Pathol. **127:** 27–37.
24. BARNETT, D., G. WILSON, A. LAWRENCE & G. BUCKLEY. 1988. The interleukin-2 receptor and its expression in the acute leukemias and lymphoproliferative disorders. Dis. Markers **6:** 133–139.
25. ROSOLEN, A., M. NAKANISHI, D. POPLACK, *et al.* 1989. Expression of interleukin-2 receptor beta subunit in hematopoietic malignancies. Blood **73:** 1968–1972.
26. KUNG, E., K. MEISSNER & T. LONING. 1988. Cutaneous T-cell lymphoma: immunocytochemical study on activation/proliferation and differentiation associated antigens in lymph nodes, skin, and peripheral blood. Virchows Arch. Am. Pathol. Anat. **413:** 539–549.
27. CRAIG, F. & P. BANKS. 1992. Detection of the alpha and beta components of the interleukin-2 receptor using immunologic techniques. Mod. Pathol. **5:** 118a (abstr).
28. RE, G.G., C. WATERS, L. POISSON, *et al.* 1996. Interleukin 2 (IL-2) receptor expression and sensitivity to diphteria fusion toxin DAB389IL-2 in cultured hematopoietic cells. Cancer Res. **56**(11): 2590–2595.
29. LEMAISTRE, C.F., C. MENEGHETTI, M. ROSENBLUM, *et al.* 1992. Phase I trial of an interleukin-2 receptor (IL-2R) fusion toxin (DAB_{486}IL-2) in hematologic malignancies expressing the IL-2 receptor. Blood **79:** 2547–2554.
30. TEPLER, I., G. SCHWARTZ, K. PARKER, *et al.* 1994. Phase I trial of an interleukin-2 toxin (DAB_{486}IL-2) in hematologic malignancies: complete response in a patient with Hodgkin's disease refractory to chemotherapy. Cancer **73:** 1276–1285.
31. HESKETH, P., P. CAGUIOA, H. KOH, *et al.* 1993. Clinical activity of a cytotoxic fusion protein in the treatment of cutaneous T cell lymphoma. J. Clin. Oncol. **11:** 1628–1690.

32. LEMAISTRE, C.F., F.E. CRAIG, C. MENEGHETTI, *et al.* 1993. Phase I trail of a 90-minute infusion of the fusion toxin DAB_{486}IL-2 in hematologic cancers. Cancer Res. **53:** 3930–3934.
33. KUZEL, T., S. ROSEN, L. GORDON, *et al.* 1993. Phase I trial of diphtheria toxin interleukin-2 fusion protein DAB_{486}IL-2: efficacy in mycosis fungoides and other non-Hodgkin's lymphomas. Leuk. Lymphoma **11:** 369–377.
34. FOSS, F., T. BORKOWSKI, M. GILLIOM, *et al.* 1994. Chimeric fusion protein toxin DAB_{486}IL-2 in refractory mycosis fungoides and the Sézary syndrome: correlation of activity and IL-2 receptor expression in a phase II study. Blood **84**(6): 1765–1774.
35. LEMAISTRE, C.F., M.N. SALEH, T.M. KUZEL, *et al.* 1998. Phase I trial of a ligand fusion-protein (DAB_{389}IL-2) in lymphomas expressing the receptor for interleukin-2. Blood **91**(2): 399-405.
36. SALEH, M.N., C.F. LEMAISTRE, T.M. KUZEL, *et al.* 1998. Antitumor activity of DAB_{389}IL-2 fusion toxin in mycosis fungoides. J. Am. Acad. Dermatol. **39**(1): 63-73.
37. DUVIC, M., T. KUZEL, E. OLSEN, *et al.* 1998. Quality of life is significantly improved in CTCL patients who responded to DAB_{389}IL-2 (ONTAK) fusion protein. Blood **92**(Suppl. 1): 624a (abstr).
38. OLSEN, E., M. DUVIC, A. FRANKEL, *et al.* 2001. Pivotal phase III study of two doses of DAB389IL2 (Ontak) for the treatment of cutaneous T-cell lymphoma. J. Clin. Oncol. 2001 **19**(2): 376–388.
39. FOSS, F., P. BACHA, K. OSANN, *et al.* 2001. Biological correlates of acute hypersensitivity events with DAB^{389}IL-2 in non-Hodgkin's lymphoma: decreased frequency and severity with steroid premedication. Clin. Lymphoma **1**(4): 298–301.
40. FOSS, F., P. BACHA, J. NICHOLS & F. CRAIG. 1999. Immunohistochemical detection of the p55 component of the high affinity IL-2 receptor alone is sufficient to predict for response to DAB_{389}IL-2. Proc. Am. Soc. Clin. Oncol. **18:** 14a (abstr).
41. LIN, J. & W. LEONARD. 1997. Signaling from the IL-2 receptor to the nucleus. Cytokine Growth Factors Rev. **8**(4): 313–332.
42. SIDELL, N., U. KUMMER, D. AFRAMIAN & S. THIERFELDER. 1997. Retinoid regulation of interleukin-2 receptors on human T-cells. Cell. Immunol. **179**(2): 116–125.
43. GORGUN, G., A. URBANO & F.M. FOSS. 1999. Enhanced cytotoxicity to DAB389IL-2 (ONTAK) in human T-cell lines by retinoic acid. Blood **94**(10): 281a.

WARNING: Only physicians experienced in the use of antineoplastic therapy and management of patients with cancer should use ONTAK (denileukin diftitox). Patients treated with denileukin diftitox must be managed in a facility equipped and staffed for cardiopulmonary resuscitation and where the patient can be closely monitored for an appropriate period based on his or her health status.

DESCRIPTION

ONTAK® (denileukin diftitox), a recombinant DNA-derived cytotoxic protein composed of the amino acid sequences for diphtheria toxin fragments A and B (Met_1-Thr_{387})-His followed by the sequences for interleukin-2 (IL-2; Ala_1-Thr_{133}), is produced in an *E. coli* expression system. ONTAK has a molecular weight of 58 kD. Neomycin is used in the fermentation process but is undetectable in the final product. The product is purified using reverse phase chromatography followed by a multistep diafiltration process.

ONTAK is supplied in single use vials as a sterile, frozen solution intended for intravenous (IV) administration. Each 2 mL vial of ONTAK contains 300 mcg of recombinant denileukin diftitox in a sterile solution of citric acid (20 mM), EDTA (0.05 mM) and polysorbate 20 (<1%) in Water for Injection, USP. The solution has a pH of 6.9 to 7.2.

CLINICAL PHARMACOLOGY

General: Denileukin diftitox is a fusion protein designed to direct the cytocidal action of diphtheria toxin to cells which express the IL-2 receptor. The human IL-2 receptor exists in three forms, low (CD25), intermediate (CD122/CD132) and high (CD25/CD122/CD132) affinity. The high affinity form of this receptor is usually found only on activated T lymphocytes, activated B lymphocytes and activated macrophages. Malignant cells expressing one or more of the subunits of the IL-2 receptor are found in certain leukemias and lymphomas including cutaneous T-cell lymphoma (CTCL)[1]. *Ex vivo* studies suggest that denileukin diftitox interacts with the high affinity IL-2 receptor on the cell surface and inhibits cellular protein synthesis, resulting in cell death within hours.

The biodistribution and excretion of radiolabeled denileukin diftitox were evaluated over 48 hours in rats. The liver and kidneys were the primary sites of distribution and accumulation of radiolabeled material outside of the vasculature. Denileukin diftitox was metabolized by proteolytic degradation. Excreted material was less than 25% of the total injected dose and consisted of low molecular weight breakdown products.

Pharmacokinetics: Pharmacokinetic parameters associated with denileukin diftitox were determined over a range of doses (3 to 31 mcg/kg/day) in patients with lymphoma. Denileukin diftitox was administered as an IV infusion following the schedule used in the clinical trials. Following the first dose, denileukin diftitox displayed 2-compartment behavior with a distribution phase (half-life approximately 2 to 5 minutes) and a terminal phase (half-life approximately 70 to 80 minutes). Systemic exposure was variable but proportional to dose. Clearance was approximately 1.5 to 2.0 mL/min/kg and the volume of distribution was similar to that of circulating blood (0.06 to 0.08 L/kg). No accumulation was evident between the first and fifth doses. Development of antibodies to denileukin diftitox has been shown to significantly impact clearance rates (see **CLINICAL STUDIES**, Immunogenicity). Gender, age, and race were introduced into a multivariate analysis with various pharmacokinetic parameters. The limited available data revealed no statistical relationships between these variables.

CLINICAL STUDIES

A randomized, double-blind study was conducted to evaluate doses of 9 or 18 mcg/kg/day in 71 patients with recurrent or persistent, Stage Ib to IVa CTCL. Entry to this study required demonstration of CD25 expression on at least 20% of the cells in any relevant tumor tissue sample (skin biopsy) or circulating cells. Tumor biopsies were not evaluated for expression of other IL-2 receptor subunit components (CD122/CD132). ONTAK was administered as an IV infusion daily for 5 days every 3 weeks. Patients received a median of 6 courses of ONTAK therapy (range 1 to 11). The study population had received a median of 5 prior therapies (range 1 to 12) with 63% of patients entering the trial with Stage IIb or more advanced stage disease. Overall, 30% (95% CI: 18-41%) of patients treated with ONTAK experienced an objective tumor response (50% reduction in tumor burden which was sustained for $\geq$ 6 weeks; Table 1). Seven patients (10%) achieved a complete response and 14 patients (20%) achieved a partial response. The overall median duration of response, measured from first day of response, was 4 months with a median duration for complete response of 9 months and for partial response of 4 months. In a Phase I/II dose-escalation study, 35 patients with Stage Ia to IVb CTCL

were treated. ONTAK was administered as an IV infusion at doses ranging from 3 to 31 mcg/kg/day, daily for 5 days every 3 weeks. The overall response rate in patients with CTCL who expressed CD25 was 38% (12 of 32 patients); the complete response rate was 16% and the partial response rate was 22%. There were no responses in 21 patients with Hodgkin's Disease.

Table 1
Response in the Phase III Double-Blind Study
Patients with CTCL

Clinical Response	9 mcg/kg/day N = 35	18 mcg/kg/day N = 36
Complete Response	3 (9%)	4 (11%)
95% Confidence Interval	2 - 23%	3 - 26%
Partial Response	5 (14%)	9 (25%)
95% Confidence Interval	5 - 30%	12 - 42%
Overall Response.	8 (23%)	13 (36%)
95% Confidence Interval	10 - 40%	21 - 54%

Immunogenicity: Prior to therapy, 39% (51/131) of lymphoma patients had low titers (<1:5) of antibody which cross-reacted with the diphtheria toxin domains of denileukin diftitox, presumably due to prior diphtheria immunization. Development of anti-denileukin diftitox antibodies was observed in 41/49 patients after a single course and in 33/34 patients after 3 cycles. Following anti-denileukin diftitox antibody formation, there was a significant increase (two to threefold) in clearance, which resulted in a decrease in mean systemic exposure of approximately 75%. Changes in clearance were related to the development of antibodies.

The antibody response in all such patients was directed against the diphtheria toxin domain. A low titer of antibodies to the IL-2 portion of the denileukin diftitox molecule also developed in approximately 50% of patients. The presence or absence of antibodies did not correlate with the risk of immediate hypersensitivity-type infusional adverse events.

INDICATIONS

ONTAK is indicated for the treatment of patients with persistent or recurrent cutaneous T-cell lymphoma whose malignant cells express the CD25 component of the IL-2 receptor (See **PRECAUTIONS**, Laboratory Tests, for CD25 expression testing). The

safety and efficacy of denileukin diftitox in patients with CTCL whose malignant cells do not express the CD25 component of the IL-2 receptor have not been examined.

CONTRAINDICATIONS

ONTAK is contraindicated for use in patients with a known hypersensitivity to denileukin diftitox or any of its components: diphtheria toxin, interleukin-2, or excipients.

WARNINGS

Acute Hypersensitivity-type Reactions: Acute hypersensitivity reactions were reported in 98 of 143 patients (69%) during or within 24 hours of ONTAK infusion; approximately half of the events occurred on the first day of dosing regardless of the treatment cycle. The constellation of symptoms included one or more of the following, defined as the incidence (%) in these 98 patients: hypotension (50%), back pain (30%), dyspnea (28%), vasodilation (28%), rash (25%), chest pain or tightness (24%), tachycardia (12%), dysphagia or laryngismus (5%), syncope (3%), allergic reaction (1%) or anaphylaxis (1%). These events were severe in 2% of patients. Management consists of interruption or a decrease in the rate of infusion (depending on the severity of the reaction); 3% of infusions were terminated prematurely and reduction in rate occurred in 4% of the infusions during the clinical trials. The administration of IV antihistamines, corticosteroids, and epinephrine may also be required; two subjects received epinephrine and 18 (13%) received systemic corticosteroids in the clinical studies. These drugs and resuscitative equipment should be readily available during ONTAK administration.

Vascular Leak Syndrome: This syndrome, characterized by 2 or more of the following 3 symptoms (hypotension, edema, hypoalbuminemia) was reported in 27% (38/143) of patients in the clinical studies. Six percent (8/143) of patients were hospitalized for the management of these symptoms. The onset of symptoms in patients with vascular leak syndrome was delayed, usually occurring within the first two weeks of infusion and may persist or worsen after the cessation of denileukin diftitox. Special caution should be taken in patients with preexisting cardiovascular disease. (See **ADVERSE REACTIONS**, Cardiovascular System).

Weight, edema, blood pressure and serum albumin levels should be carefully monitored on an outpatient basis. This syndrome is usually self-limited and treatment should be used only if clinically indicated. The type of treatment will depend on whether edema or hypotension is the primary clinical problem. Pre-existing low serum albumin levels

appear to predict and may predispose patients to the syndrome (See **PRECAUTIONS**, Laboratory Tests).

PRECAUTIONS

General: Patients should be monitored carefully for infection since patients with CTCL have a predisposition to cutaneous infection. Also, the binding of denileukin diftitox to activated lymphocytes and macrophages can lead to cell death and may impair immune function in patients.

Laboratory Tests: Prior to administration of this product, the patient's malignant cells should be tested for CD25 expression. A testing service for the assay of CD25 on skin biopsy samples is available. For information on this service call 800-964-5836.

A complete blood count and a blood chemistry panel, including liver and renal function and serum albumin levels, should be performed prior to initiation of ONTAK treatment and weekly during therapy.

Eighty-three percent (118/143) of patients with lymphoma experienced hypoalbuminemia, which was considered moderate or severe in 17% (20/118) of the affected patients. For most patients, the nadir for hypoalbuminemia occurs one to two weeks after ONTAK administration. Serum albumin levels should be monitored prior to the initiation of each treatment course. Administration of ONTAK should be delayed until serum albumin levels are at least 3.0 g/dL (see **WARNINGS**).

Drug Interactions: No clinical drug interaction studies have been conducted. However, in a single *in vivo* rodent study denileukin diftitox had no effect on P450 levels.

Carcinogenesis, Mutagenesis, Impairment of Fertility: There have been no studies to assess the carcinogenic potential of denileukin diftitox. Denileukin diftitox showed no evidence of mutagenicity in the Ames test and the chromosomal aberration assay. There have been no studies to assess the effect of denileukin diftitox on fertility.

Pregnancy Category C: Animal reproduction studies have not been conducted with ONTAK. It is also not known whether ONTAK can cause fetal harm when administered to a pregnant woman or affect reproductive capacity. ONTAK should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, and because of the potential for serious adverse reactions in nursing infants, patients receiving ONTAK should discontinue nursing.

Pediatric Use: Safety and effectiveness in pediatric patients have not been established.

Geriatric Use: Forty-nine percent (35/71) of the patients enrolled in the randomized two dose study were 65 years of age or older, and those patients had response rates similar to those seen in younger patients. The following adverse events (regardless of causality) tended to be more frequent and/or more severe in lymphoma patients who were 65 years of age or older: anorexia, hypotension, anemia, confusion, rash, nausea and/or vomiting.

ADVERSE REACTIONS

Adverse reactions are presented in Table 2. These data are based on adverse reactions observed in two clinical studies of 143 patients with lymphoma, including 105 patients with CTCL, treated at doses ranging from 3 to 31 mcg/kg/day.

All patients experienced one or more adverse events. Twenty-one percent of patients required hospitalization for drug-related adverse events; the most common reasons were evaluation of fever, management of vascular leak syndrome or dehydration secondary to gastrointestinal toxicity. Five percent of clinical adverse reactions were severe or life-threatening. The occurrence of adverse events tended to diminish in frequency after the first two courses, possibly related to antibody development.

Table 2
Adverse Reactions Occurring in Lymphoma Patients
(Frequency ≥ 5% of Patients)
N = 143 patients

Body System	Combined Term	All Grades n (%)	Grades 3 and 4 n (%)
Body as a Whole			
	Chills/fever	116 (81)	31 (22)
	Asthenia	95 (66)	31 (22)
	Infection	69 (48)	34 (24)
	Pain	69 (48)	19 (13)
	Headache	37 (26)	5 (3)
	Chest pain	34 (24)	8 (6)
	Flu-like syndrome	11 (8)	0
	Injection site reaction	11 (8)	1 (1)

Cardiovascular			
	Hypotension	52 (36)	11 (8)
	Vasodilation	31 (22)	1 (1)
	Tachycardia	17 (12)	2 (1)
	Thrombotic events	10 (7)	6 (4)
	Hypertension	9 (6)	0
	Arrhythmia	8 (6)	5 (3)
Digestive			
	Nausea/vomiting	91 (64)	20 (14)
	Anorexia	51 (36)	12 (8)
	Diarrhea	42 (29)	5 (3)
	Constipation	13 (9)	2 (1)
	Dyspepsia	10 (7)	0
	Dysphagia	9 (6)	2 (1)
Hematologic and Lymphatic			
	Anemia	26 (18)	9 (6)
	Thrombocytopenia	12 (8)	3 (2)
	Leukopenia	9 (6)	4 (3)
Metabolic and Nutritional			
	Hypoalbuminemia	118 (83)	20 (14)
	Transaminase increase	87 (61)	22 (15)
	Edema	67 (47)	22 (15)
	Hypocalcemia	24 (17)	4 (3)
	Weight decrease	20 (14)	6 (4)
	Dehydration	13 (9)	10 (7)
	Hypokalemia	9 (6)	0
Musculoskeletal			
	Myalgia	25 (17)	3 (2)
	Arthralgia	11 (8)	2 (1)
Nervous			
	Dizziness	31 (22)	1 (1)
	Paresthesia	19 (13)	2 (1)
	Nervousness	16 (11)	2 (1)
	Confusion	11 (8)	8 (6)
	Insomnia	13 (9)	4 (3)
Respiratory			
	Dyspnea	42 (29)	20 (14)
	Cough increase	37 (26)	3 (2)
	Pharyngitis	25 (17)	0
	Rhinitis	19 (13)	2 (1)
	Lung disorder	11 (8)	0
Skin and Appendages			
	Rash	48 (34)	18 (13)
	Pruritus	29 (20)	5 (3)
	Sweating	15 (10)	1 (1)
Urogenital			
	Hematuria	15 (10)	5 (3)
	Albuminuria	14 (10)	1 (1)
	Pyuria	14 (10)	1 (1)
	Creatinine increase	10 (7)	1 (1)

Hypersensitivity: (see **WARNINGS**)

Vascular Leak Syndrome: (see **WARNINGS**)

Hypoalbuminemia: (see **PRECAUTIONS,** Laboratory tests)

Infectious Complications: Infections of various types were reported by 48% (69/143) of the study population, of which 23% (16/69) were considered severe. Six of the 143 patients (4%) discontinued ONTAK therapy because of infections.

Decreased lymphocyte counts (<900 cells/μL) occurred in 34% of lymphoma patients. In general, lymphocyte counts dropped during the dosing period (Days 1 to 5) and then returned to normal by Day 15. Smaller changes and more rapid recoveries were observed with subsequent courses.

Infusion-associated Reactions: (see **WARNINGS**) There are two distinct clinical syndromes associated with ONTAK infusion, an acute hypersensitivity-type symptom complex and a flu-like symptom complex. Overall, 69% of patients had infusion-related, hypersensitivity-type symptoms; for additional information, see **WARNINGS**. A flu-like syndrome was experienced by 91% of patients within several hours to days after ONTAK infusion. The symptom complex consists of one or more of the following: fever and/or chills (81%), asthenia (66%), digestive (64%), myalgias (18%) and arthralgias (8%). In the majority of patients, these symptoms were mild to moderate and responded to treatment with antipyretics and/or anti-emetics. Antipyretics and/or anti-emetics were used to relieve flu-like symptoms; however, the usefulness of these agents in ameliorating these toxicities or as prophylactic agents to decrease the incidence of the acute, flu-like toxicities has not been prospectively studied.

Gastrointestinal: The onset of diarrhea may be delayed and the duration can be prolonged. Dehydration, usually concurrent with vomiting or anorexia, occurred in 9% of the patients. The majority of transient hepatic transaminase elevations occurred during the first course of ONTAK, were self-limited and resolved within two weeks.

Rash: A variety of rashes were reported, including generalized maculopapular, petechial, vesicular bullous, urticarial and/or eczematous with both acute and delayed onset. Antihistamines may be effective in relieving the symptoms, but more severe rashes may require the use of topical and/or oral corticosteroids.

Cardiovascular System: Two patients, both of whom had known or suspected pre-existing coronary artery disease, sustained acute myocardial infarctions while on study. Ten additional patients (7%) experienced thrombotic events. Two patients with progressive disease and multiple medical problems experienced deep vein thrombosis. Another patient sustained a deep vein thrombosis and pulmonary embolus during hospitalization for management of congestive heart failure and vascular leak syndrome. One patient with a history of severe peripheral vascular disease sustained an arterial thrombosis. Six patients experienced less severe superficial thrombophlebitis. Thrombotic events were also observed in preclinical animal studies.

Infrequent Serious Adverse Events: The following serious adverse events occurred at an incidence of less than 5%: pancreatitis, acute renal insufficiency, microscopic hematuria, hyperthyroidism and hypothyroidism.

OVERDOSAGE

There is no clinical experience with accidental ONTAK overdosage and no known antidote. At a dose of 31 mcg/kg/day, the dose-limiting toxicities were moderate-to-severe nausea, vomiting, fever, chills and/or persistent asthenia. Doses greater than 31 mcg/kg/day have not been evaluated in humans. If overdose occurs, hepatic and renal function and overall fluid balance should be closely monitored.

DOSAGE AND ADMINISTRATION

ONTAK is for intravenous (IV) use only. The recommended treatment regimen (one treatment cycle) is 9 or 18 mcg/kg/day administered intravenously for five consecutive days every 21 days. ONTAK should be infused over at least 15 minutes. If infusional adverse reactions occur (see **ADVERSE REACTIONS**), the infusion should be discontinued or the rate should be reduced depending on the severity of the reaction. There is no clinical experience with prolonged infusion times (> 80 minutes).

The optimal duration of therapy has not been determined; however, only 2% (1/51) of patients who did not demonstrate at least a 25% decrease in tumor burden prior to the fourth course of treatment subsequently responded.

Special Handling:

- ONTAK must be brought to room temperature, up to 25°C (77°F), before preparing the dose. The vials may be thawed in the refrigerator at 2 to 8°C (36 to 46°F) for not more than 24 hours or at room temperature for 1 to 2 hours. **ONTAK MUST NOT BE HEATED.**
- The solution in the vial may be mixed by gentle swirling; **DO NOT VIGOROUSLY SHAKE ONTAK SOLUTION.**
- After thawing, a haze may be visible. This haze should clear when the solution is at room temperature.
- ONTAK solution must not be used unless the solution is clear, colorless and without visible particulate matter.
- **ONTAK MUST NOT BE REFROZEN.**

Preparation and Administration:

- **USE APPROPRIATE ASEPTIC TECHNIQUE IN DILUTION AND ADMINISTRATION OF ONTAK.**
- Prepare and hold diluted ONTAK in plastic syringes or soft plastic IV bags. **DO NOT USE A GLASS CONTAINER** because adsorption to glass may occur in the dilute state.
- The concentration of ONTAK must be at least 15 mcg/mL during all steps in the preparation of the solution for IV infusion. This is best accomplished by withdrawing the calculated dose from the vial(s) and injecting it into an empty IV infusion bag. **FOR EACH 1 mL OF ONTAK FROM THE VIAL(S), NO MORE THAN 9 mL OF STERILE SALINE WITHOUT PRESERVATIVE SHOULD THEN BE ADDED TO THE IV BAG.**
- The ONTAK dose should be infused over at least 15 minutes.
- **ONTAK SHOULD NOT BE ADMINISTERED AS A BOLUS INJECTION.**
- Do not physically mix ONTAK with other drugs.
- **DO NOT ADMINISTER ONTAK THROUGH AN IN-LINE FILTER.**
- Prepared solutions of ONTAK should be administered within 6 hours, using a syringe pump or IV infusion bag.
- Unused portions of ONTAK should be discarded immediately.

HOW SUPPLIED

ONTAK is supplied as:

150 mcg/mL sterile, frozen solution (300 mcg in 2 mL) in a sterile, single-use vial
NDC 64365-503-01, 6 vials in a package.
Store frozen at or below -10°C.

REFERENCES

1. Nakase K, Kita K, Nasu K, Ueda T, Tanaka I, Shirakawa S and Tsudo M. Differential expression of interleukin-2 receptor (α and ß chain) in mature lymphoid neoplasms. Amer. J. Hematol. 1994; 46: 179-183.

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Manufactured by:
Seragen, Incorporated
Hopkinton, MA 01748
US License No. 1258

Distributed by:
Ligand Pharmaceuticals Incorporated
San Diego, CA 92121

WARNING: Only physicians experienced in the use of antineoplastic therapy and management of patients with cancer should use ONTAK (denileukin diftitox). Patients treated with denileukin diftitox must be managed in a facility equipped and staffed for cardiopulmonary resuscitation and where the patient can be closely monitored for an appropriate period based on his or her health status.

DESCRIPTION

ONTAK® (denileukin diftitox), a recombinant DNA-derived cytotoxic protein composed of the amino acid sequences for diphtheria toxin fragments A and B (Met_1-Thr_{387})-His followed by the sequences for interleukin-2 (IL-2; Ala_1-Thr_{133}), is produced in an *E. coli* expression system. ONTAK has a molecular weight of 58 kD. Neomycin is used in the fermentation process but is undetectable in the final product. The product is purified using reverse phase chromatography followed by a multistep diafiltration process.

ONTAK is supplied in single use vials as a sterile, frozen solution intended for intravenous (IV) administration. Each 2 mL vial of ONTAK contains 300 mcg of recombinant denileukin diftitox in a sterile solution of citric acid (20 mM), EDTA (0.05 mM) and polysorbate 20 (<1%) in Water for Injection, USP. The solution has a pH of 6.9 to 7.2.

CLINICAL PHARMACOLOGY

General: Denileukin diftitox is a fusion protein designed to direct the cytocidal action of diphtheria toxin to cells which express the IL-2 receptor. The human IL-2 receptor exists in three forms, low (CD25), intermediate (CD122/CD132) and high (CD25/CD122/CD132) affinity. The high affinity form of this receptor is usually found only on activated T lymphocytes, activated B lymphocytes and activated macrophages. Malignant cells expressing one or more of the subunits of the IL-2 receptor are found in certain leukemias and lymphomas including cutaneous T-cell lymphoma (CTCL)[1]. *Ex vivo* studies suggest that denileukin diftitox interacts with the high affinity IL-2 receptor on the cell surface and inhibits cellular protein synthesis, resulting in cell death within hours.

The biodistribution and excretion of radiolabeled denileukin diftitox were evaluated over 48 hours in rats. The liver and kidneys were the primary sites of distribution and accumulation of radiolabeled material outside of the vasculature. Denileukin diftitox was metabolized by proteolytic degradation. Excreted material was less than 25% of the total injected dose and consisted of low molecular weight breakdown products.

Pharmacokinetics: Pharmacokinetic parameters associated with denileukin diftitox were determined over a range of doses (3 to 31 mcg/kg/day) in patients with lymphoma. Denileukin diftitox was administered as an IV infusion following the schedule used in the clinical trials. Following the first dose, denileukin diftitox displayed 2-compartment behavior with a distribution phase (half-life approximately 2 to 5 minutes) and a terminal phase (half-life approximately 70 to 80 minutes). Systemic exposure was variable but proportional to dose. Clearance was approximately 1.5 to 2.0 mL/min/kg and the volume of distribution was similar to that of circulating blood (0.06 to 0.08 L/kg). No accumulation was evident between the first and fifth doses. Development of antibodies to denileukin diftitox has been shown to significantly impact clearance rates (see **CLINICAL STUDIES**, Immunogenicity). Gender, age, and race were introduced into a multivariate analysis with various pharmacokinetic parameters. The limited available data revealed no statistical relationships between these variables.

CLINICAL STUDIES

A randomized, double-blind study was conducted to evaluate doses of 9 or 18 mcg/kg/day in 71 patients with recurrent or persistent, Stage Ib to IVa CTCL. Entry to this study required demonstration of CD25 expression on at least 20% of the cells in any relevant tumor tissue sample (skin biopsy) or circulating cells. Tumor biopsies were not evaluated for expression of other IL-2 receptor subunit components (CD122/CD132). ONTAK was administered as an IV infusion daily for 5 days every 3 weeks. Patients received a median of 6 courses of ONTAK therapy (range 1 to 11). The study population had received a median of 5 prior therapies (range 1 to 12) with 63% of patients entering the trial with Stage IIb or more advanced stage disease. Overall, 30% (95% CI: 18-41%) of patients treated with ONTAK experienced an objective tumor response (50% reduction in tumor burden which was sustained for $\geq$ 6 weeks; Table 1). Seven patients (10%) achieved a complete response and 14 patients (20%) achieved a partial response. The overall median duration of response, measured from first day of response, was 4 months with a median duration for complete response of 9 months and for partial response of 4 months. In a Phase I/II dose-escalation study, 35 patients with Stage Ia to IVb CTCL

were treated. ONTAK was administered as an IV infusion at doses ranging from 3 to 31 mcg/kg/day, daily for 5 days every 3 weeks. The overall response rate in patients with CTCL who expressed CD25 was 38% (12 of 32 patients); the complete response rate was 16% and the partial response rate was 22%. There were no responses in 21 patients with Hodgkin's Disease.

Table 1
Response in the Phase III Double-Blind Study
Patients with CTCL

Clinical Response	9 mcg/kg/day N = 35	18 mcg/kg/day N = 36
Complete Response	3 (9%)	4 (11%)
95% Confidence Interval	2 - 23%	3 - 26%
Partial Response	5 (14%)	9 (25%)
95% Confidence Interval	5 - 30%	12 - 42%
Overall Response .	8 (23%)	13 (36%)
95% Confidence Interval	10 - 40%	21 - 54%

Immunogenicity: Prior to therapy, 39% (51/131) of lymphoma patients had low titers (<1:5) of antibody which cross-reacted with the diphtheria toxin domains of denileukin diftitox, presumably due to prior diphtheria immunization. Development of anti-denileukin diftitox antibodies was observed in 41/49 patients after a single course and in 33/34 patients after 3 cycles. Following anti-denileukin diftitox antibody formation, there was a significant increase (two to threefold) in clearance, which resulted in a decrease in mean systemic exposure of approximately 75%. Changes in clearance were related to the development of antibodies.

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	Hypotension	52 (36)	11 (8)
	Vasodilation	31 (22)	1 (1)
	Tachycardia	17 (12)	2 (1)
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Infectious Complications: Infections of various types were reported by 48% (69/143) of the study population, of which 23% (16/69) were considered severe. Six of the 143 patients (4%) discontinued ONTAK therapy because of infections.

Decreased lymphocyte counts (<900 cells/μL) occurred in 34% of lymphoma patients. In general, lymphocyte counts dropped during the dosing period (Days 1 to 5) and then returned to normal by Day 15. Smaller changes and more rapid recoveries were observed with subsequent courses.

Infusion-associated Reactions: (see **WARNINGS**) There are two distinct clinical syndromes associated with ONTAK infusion, an acute hypersensitivity-type symptom complex and a flu-like symptom complex. Overall, 69% of patients had infusion-related, hypersensitivity-type symptoms; for additional information, see **WARNINGS**. A flu-like syndrome was experienced by 91% of patients within several hours to days after ONTAK infusion. The symptom complex consists of one or more of the following: fever and/or chills (81%), asthenia (66%), digestive (64%), myalgias (18%) and arthralgias (8%). In the majority of patients, these symptoms were mild to moderate and responded to treatment with antipyretics and/or anti-emetics. Antipyretics and/or anti-emetics were used to relieve flu-like symptoms; however, the usefulness of these agents in ameliorating these toxicities or as prophylactic agents to decrease the incidence of the acute, flu-like toxicities has not been prospectively studied.

Gastrointestinal: The onset of diarrhea may be delayed and the duration can be prolonged. Dehydration, usually concurrent with vomiting or anorexia, occurred in 9% of the patients. The majority of transient hepatic transaminase elevations occurred during the first course of ONTAK, were self-limited and resolved within two weeks.

Rash: A variety of rashes were reported, including generalized maculopapular, petechial, vesicular bullous, urticarial and/or eczematous with both acute and delayed onset. Antihistamines may be effective in relieving the symptoms, but more severe rashes may require the use of topical and/or oral corticosteroids.

Cardiovascular System: Two patients, both of whom had known or suspected pre-existing coronary artery disease, sustained acute myocardial infarctions while on study. Ten additional patients (7%) experienced thrombotic events. Two patients with progressive disease and multiple medical problems experienced deep vein thrombosis. Another patient sustained a deep vein thrombosis and pulmonary embolus during hospitalization for management of congestive heart failure and vascular leak syndrome. One patient with a history of severe peripheral vascular disease sustained an arterial thrombosis. Six patients experienced less severe superficial thrombophlebitis. Thrombotic events were also observed in preclinical animal studies.

Infrequent Serious Adverse Events: The following serious adverse events occurred at an incidence of less than 5%: pancreatitis, acute renal insufficiency, microscopic hematuria, hyperthyroidism and hypothyroidism.

OVERDOSAGE

There is no clinical experience with accidental ONTAK overdosage and no known antidote. At a dose of 31 mcg/kg/day, the dose-limiting toxicities were moderate-to-severe nausea, vomiting, fever, chills and/or persistent asthenia. Doses greater than 31 mcg/kg/day have not been evaluated in humans. If overdose occurs, hepatic and renal function and overall fluid balance should be closely monitored.

DOSAGE AND ADMINISTRATION

ONTAK is for intravenous (IV) use only. The recommended treatment regimen (one treatment cycle) is 9 or 18 mcg/kg/day administered intravenously for five consecutive days every 21 days. ONTAK should be infused over at least 15 minutes. If infusional adverse reactions occur (see **ADVERSE REACTIONS**), the infusion should be discontinued or the rate should be reduced depending on the severity of the reaction. There is no clinical experience with prolonged infusion times (> 80 minutes).

The optimal duration of therapy has not been determined; however, only 2% (1/51) of patients who did not demonstrate at least a 25% decrease in tumor burden prior to the fourth course of treatment subsequently responded.

Special Handling:

- ONTAK must be brought to room temperature, up to 25°C (77°F), before preparing the dose. The vials may be thawed in the refrigerator at 2 to 8°C (36 to 46°F) for not more than 24 hours or at room temperature for 1 to 2 hours. **ONTAK MUST NOT BE HEATED.**
- The solution in the vial may be mixed by gentle swirling; **DO NOT VIGOROUSLY SHAKE ONTAK SOLUTION.**
- After thawing, a haze may be visible. This haze should clear when the solution is at room temperature.
- ONTAK solution must not be used unless the solution is clear, colorless and without visible particulate matter.
- **ONTAK MUST NOT BE REFROZEN.**

Preparation and Administration:

- **USE APPROPRIATE ASEPTIC TECHNIQUE IN DILUTION AND ADMINISTRATION OF ONTAK.**
- Prepare and hold diluted ONTAK in plastic syringes or soft plastic IV bags. **DO NOT USE A GLASS CONTAINER** because adsorption to glass may occur in the dilute state.
- The concentration of ONTAK must be at least 15 mcg/mL during all steps in the preparation of the solution for IV infusion. This is best accomplished by withdrawing the calculated dose from the vial(s) and injecting it into an empty IV infusion bag. **FOR EACH 1 mL OF ONTAK FROM THE VIAL(S), NO MORE THAN 9 mL OF STERILE SALINE WITHOUT PRESERVATIVE SHOULD THEN BE ADDED TO THE IV BAG.**
- The ONTAK dose should be infused over at least 15 minutes.
- **ONTAK SHOULD NOT BE ADMINISTERED AS A BOLUS INJECTION.**
- Do not physically mix ONTAK with other drugs.
- **DO NOT ADMINISTER ONTAK THROUGH AN IN-LINE FILTER.**
- Prepared solutions of ONTAK should be administered within 6 hours, using a syringe pump or IV infusion bag.
- Unused portions of ONTAK should be discarded immediately.

HOW SUPPLIED

ONTAK is supplied as:

150 mcg/mL sterile, frozen solution (300 mcg in 2 mL) in a sterile, single-use vial
NDC 64365-503-01, 6 vials in a package.
Store frozen at or below -10°C.

REFERENCES

1. Nakase K, Kita K, Nasu K, Ueda T, Tanaka I, Shirakawa S and Tsudo M. Differential expression of interleukin-2 receptor (α and ß chain) in mature lymphoid neoplasms. Amer. J. Hematol. 1994; 46: 179-183.

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1: Breast Cancer Res Treat. 1994;32(1):97-103.

Targeting the EGF receptor in breast cancer treatment.

LeMaistre CF, Meneghetti C, Howes L, Osborne CK.

South Texas Cancer Institute, San Antonio 78229.

Immunotoxins are a relatively new class of cytotoxic agents consisting of a catalytic toxin linked to an appropriate targeting ligand. The ligand directs th toxin to the surface of a tumor cell, whereupon the toxin enters the cell and catalytically inactivates the ribosome, thus disrupting protein synthesis and effecting cell death. Monoclonal antibodies (or their fragments) have been m commonly used to carry chemically conjugated toxins to proteins or antigens overexposed on the tumor cell surface, but specific ligands for tumor cell sur receptors could also provide effective targeting. The receptor for epidermal growth factor (EGFR) is overexpressed primarily in poor prognosis breast ca that do not respond well to traditional therapies. Because EGFR is frequently overexpressed in breast cancer tissue and is associated with a poor prognosis, an attractive target for antitumor therapy. DAB389EGF is an EGFR specific fusion toxin produced with recombinant DNA techniques consisting of seque for the enzymatically active and membrane translocation domains of diphthe toxin plus sequences for human epidermal growth factor. DAB389EGF is a potent, EGFR specific, cytotoxic agent which rapidly inhibits protein synthes a mechanism of action similar to that of diphtheria itself. Preclinical studies i laboratory and in animals now suggest the feasibility of investigating such ar agent in the targeted therapy of patients with human breast cancer.

Publication Types:

- Review
- Review, Tutorial

PMID: 7819590 [PubMed - indexed for MEDLINE]

Recombinant heregulin-Pseudomonas exotoxin fusion proteins: interactions with the heregulin receptors and antitumor activity vivo.

Yang D, Kuan CT, Payne J, Kihara A, Murray A, Wang LM, Alimandi Pierce JH, Pastan I, Lippman ME.

Lombardi Cancer Center, Department of Biochemistry and Molecular Biolog Georgetown University Medical Center, Washington, DC 20007, USA.

Growth factor receptors provide unique opportunities for development of tar anticancer therapy. Members of the type I receptor tyrosine kinase family, including epidermal growth factor (EGF) receptor (EGFR) and ErbB-2/neu, a often overexpressed in various human cancer cells, including breast. Recentl has been shown that both ErbB-3 and ErbB-4 are receptors for heregulin (HRG)/Neu differentiation factor. Eight chimeric toxins composed of the extracellular and EGF-like domains of four different HRG isoforms and trun Pseudomonas exotoxin (PE38KDEL) were constructed. The fusion proteins exhibited activity similar to the native HRG in inducing ErbB receptors phosphorylation. The EGF-like domain of HRG13 and HRGbeta2 fused to PE38KDEL showed the highest cytotoxic activity, with a IC50 of < or = 0.0(ng/ml. The alpha isoforms that were fused to PE38KDEL were 100-fold less active than the beta isoforms. The HRG-Pseudomonas exotoxin (PE) toxins s extremely high activity against cells expressing ErbB-4 receptor, alone or together with other members of the ErbB receptor family. Cells that do not express ErbB-4 but express ErbB-3 receptor, together with the ErbB-2 or EG exhibited moderate sensitivity to HRG-PE toxins. HRG-PE toxins have little no activity against cells expressing EGFR, ErbB-2, or ErbB-3 alone. More th an 80% tumor regression was achieved by intratumor injection of 1 microg o fusion proteins per day for 5 days. Continuous i.p. administration of EGF-lik domain of HRGbeta1-PE38KDEL for 7 days via a miniosmotic pump at a dc 40 microg/kg/day inhibited the growth of ErbB-4 receptor positive but not E1 receptor negative cell lines in athymic nude mice. We conclude that there is therapeutic potential of HRG-PE toxins in the therapy of cancers overexpress the ErbB-4 or ErbB-2 plus ErbB-3 receptors.

PMID: 9563895 [PubMed - indexed for MEDLINE]

1: Int J Cancer. 1998 Sep 25;78(1):106-11.

Targeting tumor cells via EGF receptors: selective toxicity of an HBEGF-toxin fusion protein.

Chandler LA, Sosnowski BA, McDonald JR, Price JE, Aukerman SL, B A, Pierce GF, Houston LL.

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Over-expression of the epidermal growth factor receptor (EGFR) is a hallma numerous solid tumors, thus providing a means of selectively targeting therapeutic agents. Heparin-binding epidermal growth factor (HBEGF) binds EGFRs with high affinity and to heparan sulfate proteoglycans, resulting in increased mitogenic potential compared to other EGF family members. We h investigated the feasibility of using HBEGF to selectively deliver a cytotoxic protein into EGFR-expressing tumor cells. Recombinant fusion proteins consisting of mature human HBEGF fused to the plant ribosome-inactivating protein saporin (SAP) were expressed in Escherichia coli. Purified HBEGF-S chimeras inhibited protein synthesis in a cell-free assay and competed with E for binding to receptors on intact cells. A construct with a 22-amino-acid flex linker (L22) between the HBEGF and SAP moieties exhibited an affinity for EGFR that was comparable to that of HBEGF. The sensitivity to HBEGF-L2 SAP was determined for a variety of human tumor cell lines, including the 6(lines comprising the National Cancer Institute Anticancer Drug Screen. HBE L22-SAP was cytotoxic in vitro to a variety of EGFR-bearing cell lines and inhibited growth of EGFR-over-expressing human breast carcinoma cells in v In contrast, the fusion protein had no effect on small-cell lung carcinoma cell which are EGFR-deficient. Our results demonstrate that fusion proteins comp of HBEGF and SAP exhibit targeting specificity and cytotoxicity that may b therapeutic value in treating a variety of EGFR-bearing malignancies.

PMID: 9724101 [PubMed - indexed for MEDLINE]

1: Protein Eng. 1998 Dec;11(12):1285-92.

Human pancreatic RNase1-human epidermal growth factor fusi an entirely human 'immunotoxin analog' with cytotoxic propert against squamous cell carcinomas.

Psarras K, Ueda M, Yamamura T, Ozawa S, Kitajima M, Aiso S, Komai S, Seno M.

Department of Surgery, School of Medicine, Keio University, Tokyo, Japan.

The gene encoding human pancreatic ribonuclease 1 (hpRNasel) was fused w gene encoding human epidermal growth factor (hEGF). The hybrid human protein was isolated from Escherichia coli inclusion bodies, refolded and pur to homogeneity. The fusion protein competed with 125I-hEGF for binding to hEGF receptors (EGFR) and had ribonucleolytic activities approaching those hpRNase1. Several conformations having different enzymatic activities coul detected after reversed-phase high-performance liquid chromatographic anal the less hydrophobic molecules being the most active. The hybrid protein wa specifically cytotoxic to A431, an EGFR overexpressing squamous carcinom cell line, with an IC50 of approximately 10(-7) M. In contrast, recombinant hpRNase1 had an IC50 higher than 10(-4) M. A mixture of free hEGF and fr hpRNasel was not more cytotoxic than hpRNasel alone and no cytotoxicity w detected in EGFR-deficient control cells. Taken together, these data suggest t this construct might be useful for targeted therapy of esophageal, lung and ot squamous cell carcinomas and also breast cancers overexpressing EGFR, wh correlate with a poor prognosis and cannot be cured by surgery alone. Engineering hybrid molecules with endogenous human proteins for targeted therapy may alleviate the dose-limiting immunogenicity and toxicity of conventional immunotoxins.

PMID: 9930679 [PubMed - indexed for MEDLINE]

1: Life Sci. 1999;64(16):1435-45.

Cloning and cytotoxicity of fusion proteins of EGF and angioger

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Department of Microbial Chemistry, College of Pharmacy, Seoul National University, Kwanak-Gu, South Korea.

Targeted toxins represent a new approach to specific cytocidal therapy. Immunotoxins based on plant and microbial toxins are very immunogenic. T develop a targeted therapy that is less immunogenic and easily invades target tissues, four fusion proteins containing human angiogenin targeted by human EGF have been constructed. EGF is a single chain polypeptide, which binds epidermal growth factor receptor (EGFR) and is known to be internalized by endocytosis. Angiogenin has been separately fused either at the amino termir or the carboxyl terminus of EGF via linkers, giving rise to angiogenin-gly-EC angiogenin-(gly)4ser-EGF and EGF-angiogenin, EGF-gly-angiogenin, respectively. The fusion proteins were over-expressed in Escherichia coli anc purified from periplasmic eluents by affinity chromatography. EGF-angioger and EGF-gly-angiogenin maintained receptor-binding activity of EGF and R activity of angiogenin in a single peptide and actively inhibited growth of hu EGFR-positive target cells in culture. They are expected to have a very low immunogenic potential in humans because of their endogenous origin and als have another potential therapeutic advantage because these fusion proteins m have overcome conventional immunotoxin and possess increased ability to penetrate because of their small size.

PMID: 10321723 [PubMed - indexed for MEDLINE]

A Murine Cytokine Fusion Toxin Specifically Targeting the Murine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor on Normal Committed Bone Marrow Progenitor Cells and GM-CSF–Dependent Tumor Cells

By Chung-Huang Chan, Bruce R. Blazar, Cindy R. Eide, Robert J. Kreitman, and Daniel A. Vallera

A fusion protein was synthesized consisting of the murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) gene spliced to a truncated form of the diphtheria toxin (DT_{390}) gene coding for a molecule that retained full enzymatic activity, but excluded the native binding domain. The DT_{390}-mGM-CSF hybrid gene was cloned into a vector under the control of an inducible promoter and the protein expressed in *Escherichia coli*. After induction, a protein was purified from inclusion bodies in accord with the deduced molecular weight of DT_{390} mGM-CSF. Cell-free studies of the adenosine diphosphate–ribosylating activity of DT_{390} mGM-CSF showed results that were similar to those of native DT. The DT_{390} mGM-CSF immunotoxin inhibited FDCP2.1d, a murine myelomonocytic tumor line expressing the GM-CSF receptor with an IC_{50} (concentration inhibiting 50% activity) of 5×10^{-11} mol/L. The fusion toxin was specifically cytotoxic and directed by the GM-CSF portion of the molecule because addition of a monoclonal antibody directed against GM-CSF inhibited its ability to kill the cell line. Cell lines that do not express GM-CSF receptor were not inhibited by the fusion toxin. DT_{390} mGM-CSF was also able to specifically inhibit normal committed bone marrow (BM) progenitor cells as measured in clonal colony-forming unit granulocyte-macrophage assays. Together, these findings indicate that DT_{390} mGM-CSF may be useful as a novel tool for purging BM of contaminating leukemia cells or in vivo for eliminating residual leukemia cells. Also, it can be used to determine whether committed and/or noncommitted BM progenitor cells express the GM-CSF receptor.

MURINE granulocyte-macrophage colony-stimulating factor (mGM-CSF) is composed of 124 amino acids with a calculated molecular weight of 14.138 kD.[1] GM-CSF can stimulate proliferation and differentiation of colony-forming unit–granulocyte-macrophage (CFU-GM) progenitor cells as well as enhance the function of mature neutrophils, monocytes, and eosinophils.[2] Exposure of CFU-GM progenitors to GM-CSF causes rapid entry of cells into the cell cycle. The biologic actions of GM-CSF are mediated by binding to a specific high-affinity receptor, consisting of two components designated as α and β subunits.[3,4] These GM-CSF receptors are found on the surface of myeloid precursors, granulocytes, mononuclear phagocytes,[5-7] and also frequently present on the myeloid malignancies.[8-10] In fact, the majority of cases of acute myeloid leukemia express high-affinity receptors for GM-CSF.

GM-CSF can interact with myeloid leukemic cells because GM-CSF, either by itself or in association with other cytokines, can induce proliferation of myeloid clonogenic blasts.[11-13] Several reports suggest that GM-CSF receptors are expressed on human nonhematopoietic tumor cell lines[14-17] and GM-CSF is capable of stimulating the growth of human solid tumor cell lines including those derived from small cell lung carcinoma, melanoma, renal carcinoma, colon carcinoma, gastric carcinoma, and ovarian carcinoma.[14,18-20]

The first step in the proliferative action of GM-CSF on leukemic progenitors is ligand binding to specific membrane receptors.[5,6,21,22] The receptor is composed of two subunits and the binding of these α and β subunits together form a high-affinity (dissociation constant [kd], approximately 40 pmol/L) receptor complex.[5] The binding of GM-CSF to this receptor causes rapid internalization of the ligand-receptor complex.[23] Because of the internalization of GM-CSF, we reasoned that GM-CSF could serve as a ligand for delivering a toxic molecule such as diphtheria toxin (DT) to myeloid leukemic cells.

DT is a well-studied glycoprotein with a molecular weight of 58 kD. DT has potent cell-killing ability and requires internalization.[24] Its mechanism involves adenosine diphosphate (ADP)-ribosylation of elongation factor-2, resulting in inhibition of cellular protein synthesis and death of the cell. Investigators have shown that DT induces DNA degradation and morphologic changes consistent with apoptosis.[25] Delivering a single DT molecule into the cytoplasm is sufficient to kill a cell.[26] Native DT contains three domains: the cell-binding domain, the translocation domain, and the enzymatic cytotoxic domain.[26-28] The cell-binding domain of the DT gene can be replaced by a growth factor gene, resulting in a toxin-growth factor hybrid gene, whose protein product is targeted to a specific growth-factor receptor. Fusion toxins have been reported that specifically target DT to cytokine receptors including interleukin-2 (IL-2), IL-4, IL-6, and G-CSF receptors.[29-33]

We constructed a fusion toxin targeting cells bearing the mGM-CSF receptor (1) to devise a reagent that is potentially useful in destroying the residual myeloid leukemic cells dur-

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plates in a volume of 200 μL. One-microcurie [H³]-thymidine and exogenous mGM-CSF at final concentration of 1 ng/mL were added into each well. After a 24-hour incubation, the cells were procured on glass fiber filters. Filters were washed, dried, and counted according to standard methods suggested by manufacturer. Cells cultured with media alone served as the control. All assays were performed in triplicate. Two additional control murine cell lines EL4, a T-cell leukemia/lymphoma and the myeloid leukemia C1498 (American Type Culture Collection, Rockville, MD) were used that did not respond to mGM-CSF.

Colony-forming assay. DT_{390} mGM-CSF, DT_{390} mIL-2, and native DT were examined for their effects on BM cells in a colony-forming assay, which was performed by short-term culture of toxin-treated murine BM cells in complete methylcellulose media (30% fetal calf serum, 1% pokeweed mitogen–stimulated murine spleen cell conditioned medium, 1% bovine serum albumin (BSA), 0.9% methylcellulose, 10^{-4} mol/L 2-mercaptoethanol, and 3 U/mL erythropoietin) (StemCell Technologies, Vancouver BC, Canada). Briefly, BM cells were collected into RPMI 1640 media by flushing the shafts of femora and tibia of C57BL/6 mice. Cells were resuspended at 5×10^4 cells/mL in complete methylcellulose media with toxin at a final concentration of either 10^{-8} or 10^{-9} mol/L and were plated and incubated in culture dishes for 14 days under fully humidified conditions in an atmosphere of 5% CO_2 at 37°C. Under an inverted microscope, colonies of greater than 50 cells were scored as CFU-GM according to their morphology. All assays were performed in duplicate.

RESULTS

Genetic construction of DT_{390} mGM-CSF. The DNA fragments encoding the structural gene for DT_{390} and mGM-CSF were obtained by separate PCRs with the sizes of 1,239 and 380 bp, respectively. After the third PCR, the resulting SOE product, DT_{390} mGM-CSF hybrid gene, was generated with 1,601-bp size. The DT_{390} mGM-CSF hybrid gene encodes an *Nco* I restriction site, an ATG initiation codon, the first 389 amino acids of DT, a 20–amino acid interchain spacer, the mature murine GM-CSF polypeptide, and an *Xho* I restriction site. After digestion, the DT_{390}–mGM-CSF hybrid gene was cloned into the pET21d plasmid under the control of the IPTG-inducible T7 promoter to create pDT GM-CSF. Restriction endonuclease digestion and DNA sequencing analysis were used to verify that DT_{390} mGM-CSF hybrid gene sequence had been cloned in frame (data not shown).

The plasmid was sequenced by the University of Minnesota Microchemical Facility (University of Minnesota, Minneapolis). The junctional region was found to encode for amino acids 333 to 389 of DT with one substitution of alanine at postion 356 by serine. The linker was found to encode for the following amino acids, $[(Gly)_4Ser]_2GlyAla(Gly)_2Ser(Gly)_4SerPhe$ joined by a sequence encoding amino acids 27 through 175 of mGM-CSF.

Expression and purification of DT_{390}–mGM-CSF fusion protein. Expression of the fusion protein in *E coli* was induced with IPTG at 37°C. Coomassie blue–stained SDS-polyacrylamide gel of whole bacterial lysate post-IPTG induction showed a protein migrating at 58 kD, which corresponds to the expected size for DT_{390}–mGM-CSF protein. The localization study of the expressed fusion protein showed that DT_{390} mGM-CSF was retained in the inclusion bodies (Fig 2). To extract the DT_{390}–mGM-CSF protein, the inclusion bodies were isolated, denatured, and refolded as described in Materials and Methods. After the renaturation procedure, the crude DT_{390} mGM-CSF was purified by sequential chromatography. The elution from the anion-exchange Q-sepharose column showed an enrichment of a protein with an electrophoretic mobility corresponding to an apparent molecular mass of 58 kD (Fig 2, lane 6). To further purify this fusion protein, pooled peak fractions from the anion-exchange Q-sepharose column were subjected to high-performance liquid chromatography (HPLC) using a TSK-250 sizing column (Fig 2, lane 7). The final product was 80% pure. To prove that the protein was indeed a fusion of of this DT_{390} spliced to mGM-CSF, further analysis was performed by immunoblotting. Anti-DT antiserum was able to recognize the DT_{390} mGM-CSF, both in the crude bacterial lysate and after renaturation (Fig 3). It is of interest to note that anti–mGM-CSF antiserum was able to recognize the DT_{390} mGM-CSF only after proper renaturation.

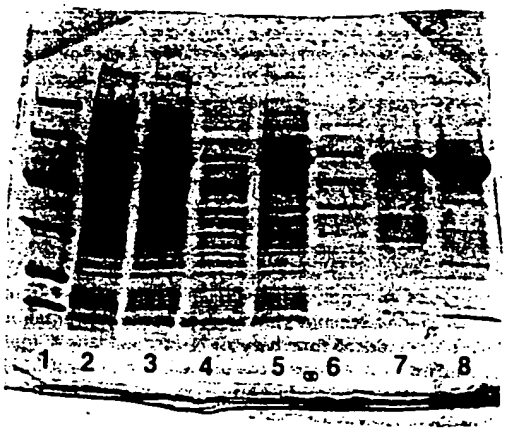

Fig 2. SDS-PAGE of the purified DT_{390}–mGM-CSF protein stained with Coomassie Blue. Lane 1, molecular weight standard; lane 2, uninduced total bacteria lysate; lane 3, induced total bacteria lysate; lane 4, fraction of soluble protein in cytoplasm; lane 5, fraction of insoluble protein in inclusion body; lane 6, soluble protein in periplasmic space; lane 7, major elution of anion-exchange column; and lane 8, major peak of HPLC sizing column. The molecular standards migrated (beginning with the largest protein) at 97, 66, 45, 31, 22, and 14 kD.

Enzymatic activity and in vitro cytotoxicity. Protein synthesis inhibition by DT is caused by fragment A–catalyzed ADP-ribosylation of cytoplasmic EF-2. To determine whether the DT_{390}–mGM-CSF protein also displays such enzymatic activity, a cell-free assay system was used, in which rabbit reticulocyte lysate, a source of EF-2, was exposed to either native DT or DT_{390} mGM-CSF in the presence of [^{32}P]-nicotinamide adenine dinucleotide. Incubation with either toxin showed a similar dose-dependent increase in [^{32}P] incorporation into the TCA-precipitable fraction (Fig 4).

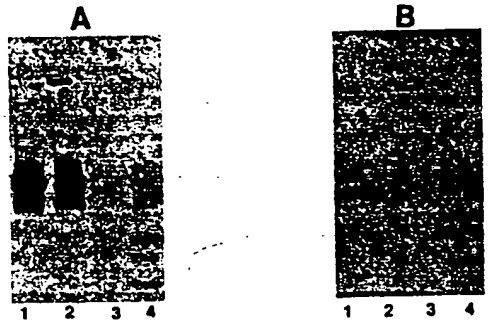

Fig 3. Western blot analysis of DT_{390}–mGM-CSF protein. (A) Nitrocellulose probed with polyclonal anti-DT serum. Lane 1, renatured protein after anion-exchange column; lane 2, induced total bacteria lysate; lane 3, uninduced total bacteria lysate; and lane 4, molecular weight standards. (B) Nitrocellulose probed with anti–mGM-CSF antibody. Lane 1, renatured protein after anion-exchange column; lane 2, induced total bacteria lysate; lane 3, uninduced total bacteria lysate; and lane 4, molecular weight standards.

This result confirmed that DT_{390} mGM-CSF possesses ADP-ribosyl transferase activity.

To characterize the cytotoxic activity of DT_{390} mGM-CSF, a bioassay was devised using the mGM-CSF–dependent myelomonocytic leukemia cell line FDCP2.1d. The cytotoxicity was evaluated by measuring the inhibition of cellular proliferation. The ability of various concentrations of DT_{390} mGM-CSF to inhibit the proliferation on FDCP2.1d cells was examined. FDCP2.1d cells were inhibited by DT_{390} mGM-CSF in a dose-dependent manner with an IC_{50} of 5×10^{-11} mol/L or 3 ng/mL (Fig 5). To determine if the cytotoxic activity of DT_{390} mGM-CSF on FDCP2.1d cells was mediated by the binding of the mGM-CSF moiety, several other toxins including DT_{390} hIL-2, DT_{390} mIL-4, and native DT were

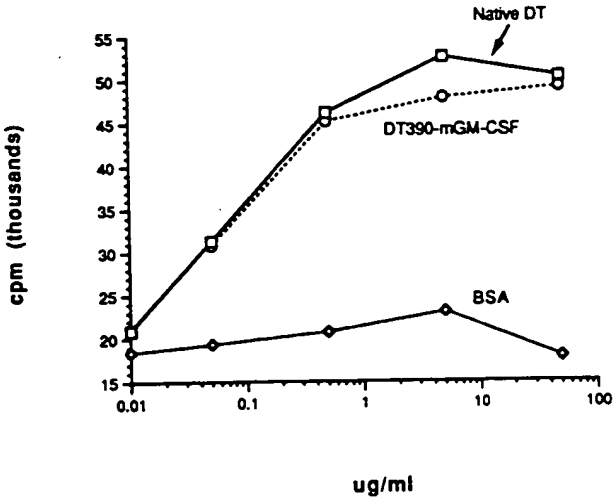

Fig 4. ADP ribosyl transferase activity of DT_{390} mGM-CSF. Trypsin-nicked DT_{390} mGM-CSF, trypsin-nicked native diphtheria toxin, or BSA was studied in cell-free assay. Protein was added at various concentration to the reaction system. The activity was measured as the count of bound ^{32}P-ADP-ribose to rabbit reticulocyte lysate (EF-2).

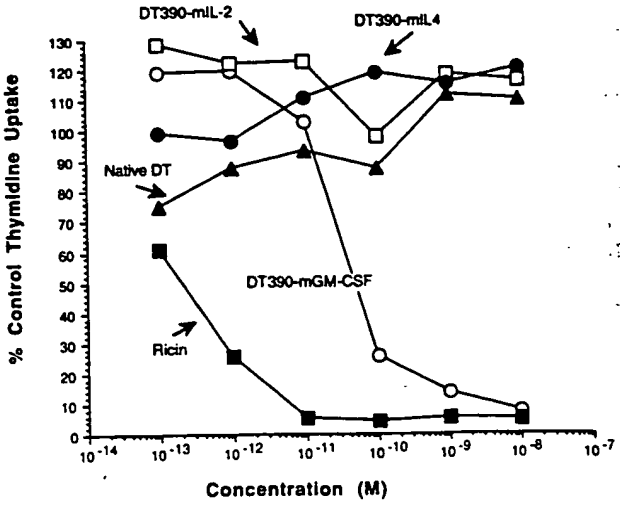

Fig 5. Cytotoxic activity of fusion toxins on FDCP2.1d cells. Fusion toxins were added at various concentrations to FDCP2.1d cells for 4 hours. After washing out toxin, the percent incorporation of [H^3] thymidine relative to untreated controls was determined.

also assayed for their activity against FDCP2.1d. In contrast with DT_{390} mGM-CSF, FDCP2.1d cells were resistant to DT_{390} hIL-2, DT_{390} mIL-4, and native DT up to a concentration of 1×10^{-8} mol/L. Furthermore, anti–mGM-CSF antibodies blocked the cytotoxic effect of DT_{390} mGM-CSF in a dose-dependent manner . Without addition of anti–mGM-CSF antibodies, DT390 mGM-CSF at concentration of 1×10^{-9} mol/L produced a 84% inhibition of cellular proliferation of FDCP2.1d. The addition of 1 nmol/L anti–mGM-CSF antibodies partially neutralized this cytotoxic effect. The addition of 10 nmol/L or 100 nmol/L anti–mGM-CSF antibodies completely neutralized this cytotoxic effect (Fig 6). The addition of 10 nmol/L GM-CSF to 1 nmol/L DT_{390}

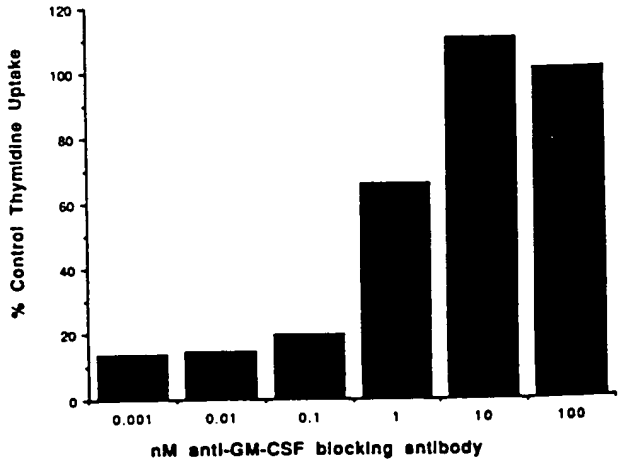

Fig 6. Neutralization of DT_{390} mGM-CSF by anti–mGM-CSF antibody. DT_{390} mGM-CSF (10^{-9} mol/L) was incubated with various concentrations of anti–mGM-CSF or with no antibody and added to FDCP2.1d cells. Results are expressed as the percent of untreated control cells.

mGM-CSF inhibited the response 25%, but the addition of IL-3 did not inhibit. The effect of DT_{390} mGM-CSF on two GM-CSF nonresponsive cell lines was performed to further examine the specificity of the cytotoxic effect. DT_{390} mGM-CSF did not inhibit the T-cell line EL4 or leukemia cell line C1498 (Table 2). Together these data indicate that DT_{390} mGM-CSF is specifically cytotoxic to cells via the GM-CSF ligand-receptor complex.

The effect of DT_{390} mGM-CSF on myeloid progenitor stem cells. It has been well documented that GM-CSF plays a role in the development of the myeloid lineage in hematopoiesis. To test the effect of DT_{390} mGM-CSF on committed myeloid progenitor cells, a colony-forming assay was performed by incubating murine BM cells with toxins including DT_{390} mGM-CSF, DT_{390} hIL-2, and native DT. The DT_{390} mGM-CSF inhibited the formation of CFU-GM up to 90% at the toxin concentrations of 1 nmol/L and 10 nmol/L. In contrast, DT_{390} mIL-2 and native DT had little inhibitory effect on CFU-GM (Fig 7). From these data, we conclude that DT_{390} mGM-CSF has activity against committed myeloid progenitor cells in vitro.

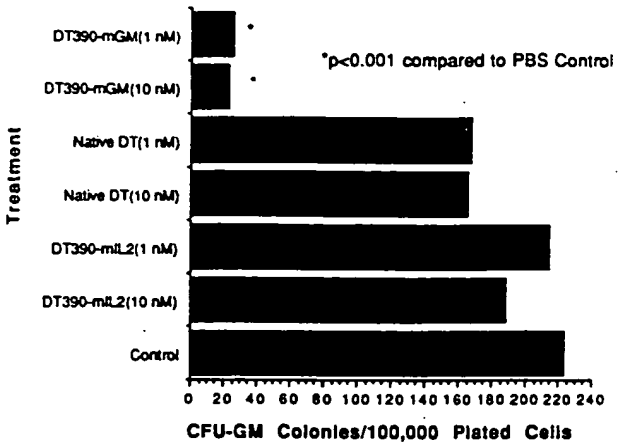

Fig 7. Effect of DT_{390} mGM-CSF on the colony formation of CFU-GM myeloid progenitor cells. Mouse BM cells were incubated with fusion toxin and BM was cultured. Colony number was determined relative to 1×10^5 mononuclear BM cells plated on the semisolid methylcellulose media.

DISCUSSION

The unique contribution of this work is the construction and description of a fusion toxin, DT_{390} mGM-CSF by genetically splicing the DNA segment encoding the ADP-ribosyl transferase enzymatic and hydrophobic translocation enhancing region of DT, but not the native binding site to the DNA segment encoding the amino acids of the mature mGM-CSF molecule. Our data show that the selective binding of this chimeric protein to GM-CSF receptor expressing myelomonocytic leukemia cell lines results in the delivery of a potent and fatal signal that precipitously decreases the proliferation of these cells.

The potency of DT_{390} mGM-CSF was high because we measured an IC_{50} of about 5×10^{-11} mol/L against the cell line FDCP2.1d. Recently, Lappi et al has chemically conjugated human GM-CSF to the ribosome-inactivating protein saporin (SAP).[37] The hGM-CSF-SAP showed an IC_{50} about 3 to 4×10^{-12} mol/L on cell lines transfected with both subunits of GM-CSF receptors. Although these studies involved different toxin moieties, different species of GM-CSF, and different receptor numbers on different cell lines, DT_{390} mGM-CSF was comparable in cytotoxicity to this and other diphtheria-based fusion toxins. For example, DAB_{389} IL-2 and DAB_{486} IL-2 usually show potency with IC_{50} ranging from 2×10^{-11} to 1×10^{-10} mol/L.[38,39] DT_{390} mGM-CSF was specific because it was cytotoxic to mGM-CSF dependent cell line FDCP2.1d, but not the mGM-CSF nonresponsive cell lines C1498 and EL4. DT_{390} mGM-CSF activity was inhibited by an excess of anti–mGM-CSF antibody and, thus, was directed by the GM-CSF portion of the molecule. The IC_{50} of the fusion protein (50 pmol/L) corresponded to the reported dissociation constant for high-affinity GM-CSF binding sites (20 to 60 pmol/L).[40]

Table 2. Sensitivity of Various Cell Lines to DT_{390}-mGM-CSF

Cell Line	Origin	mGM-CSF Response	IC_{50} (mol/L)
FDCP2.1d	Mouse myelomonocytic leukemia	mGM-CSF dependent	5×10^{-11}
C1498	Mouse lymphoma	Negative	$1 \times >10^{-8}$
EL4	Mouse T-cell lymphoma	Negative	$1 \times >10^{-8}$

Cell lines were incubated with DT_{390}-mGM-CSF for 4 hours, washed, pulsed with tritiated thymidine, and then incubated for 24 hours. Cells were harvested onto filters and then counted. Data were plotted as percent control inhibition versus increasing concentration of fusion toxin. IC_{50} dosage (the dose at which 50% of the total response was inhibited) was determined from the curves.

The study was initiated because alternative therapies are still a priority for the treatment of acute nonlymphocytic leukemias, which are still a serious clinical problem. GM-CSF was selected as a ligand because GM-CSF receptor is expressed on these myeloid leukemias.[5-7] It has been postulated that GM-CSF is involved in malignant transformation and metastases because of its expression on neoplastic cells.[12,22,41] Binding GM-CSF causes internalization of GM-CSF receptor in a variety of murine cells at 37°C by receptor-mediated endocytosis.[11,40] Thus, we expected that the GM-CSF component of the fusion toxin would be able to bind to the GM-CSF receptor, resulting in the internalization of the GM-CSF receptor-fusion toxin complex into the endocytic vesicles in a fashion analogous to diphtheria toxin itself.[24,42] Our findings support this.

One major problem in myeloid leukemia is purging occult leukemia cells from patient BM before autologous BM transplant. Complete remission can be induced by intensive chemotherapy, but remission can be short-lived and the patient frequently experiences relapse of their underlying disease. Autologous BM transplantation is being explored as treatment modality designed to improve relapse-free survival. The patient's own BM is removed and then returned as a hematopoietic rescue procedure after aggressive chemotherapy and irradiation therapy. Because the procedure is complicated by metastatic leukemia cells infiltrating the BM, one

of the most commonly used techniques involve chemical purging of the BM to eliminate leukemia cells.[43] However, one drawback is that clinical chemical purging has a broad spectrum of depletion and eliminates beneficial cells including lymphocytes.[44] An advantage of using mGM-CSF to direct toxin is that GM-CSF would bind to myeloid leukemia cells[45] and not to cells that do not express the GM-CSF receptor.

Because residual metastatic leukemia cells that survive the preparative regimen can also lead to relapse and transplantation failure, DT_{390} mGM-CSF could be used for in vivo therapy, especially because there is clinical precedence for the use of DT-based fusion toxins for therapy of leukemia.[46] Although the in vivo efficacy of this agent will depend on its ability to access leukemia cells, their are several complicating issues that must also be explored. These issues can best be studied, and in some cases can only be studied, in animal models.

The issues are as follows: (1) In vivo depletion of myeloid cells might result in immunosuppression. Cells of myeloid origin participate as antigen presenting cells in generating optimal T-cell responses.[47] A fusion toxin directed against these cells might either reduce the immune response to tumor or render the host susceptible to secondary infections. (2) GM-CSF receptor expression on stem cells might limit the in vivo antileukemia effectiveness of DT_{390} mGM-CSF resulting in life-threating myelosuppression. The GM-CSF receptor is expressed on committed murine progenitor cells.[48] In fact, studies in humans show that GM-CSF is active in stimulating $CD34^+$ human progenitor cells.[49] In this paper, DT_{390} mGM-CSF was reactive against committed myeloid BM progenitors in in vitro CFU-GM assays. In separate studies (data not shown), we found that the fusion toxin had little effect against day 8 CFU-S, which measures earlier erythroid and myeloid stem cells, and day 12 CFU-S, which measures multilineage progenitor cells.[50,51] Thus, expression of mGM-CSF receptors occurs between CFU-S and CFU-GM stages of development. In future studies, DT_{390} mGM-CSF can be used in murine in vivo adoptive transfer experiments designed to directly determine stem cell expression of the GM-CSF receptor. Such experiments cannot be performed in humans. (3) Some believe that GM-CSF expression on endothelial cells could result in fusion toxin injury to the vasculature causing vascular leak sydrome, which has been problematic in the use of other immunotoxins.[52] GM-CSF stimulates the proliferation of endothelial cells.[53] However, our histopathologic studies of mice given in vivo DT_{390} mGM-CSF showed endothelialitis in some larger vessels, but no evidence of endothelial cell destruction (data not shown). (4) GM-CSF receptors are heterogenously expressed which might effect the efficacy of DT_{390} mGM-CSF against leukemic targets in vivo. Myeloid cells in mice appear to express two distinct types of GM-CSF receptors with high and low affinities, with kd of 20 to 60 pmol/L and 700 to 1,200 pmol/L, respectively.[40] The onset of proliferation of GM progenitor cells remains highly asynchronous, which may result from this heterogeneous expression of either high or low affinity GM-CSF receptors.

In conclusion, these findings describe a new fusion protein with high potential to eradiacate tumor cells of myeloid lineage. There is potential for using this agent to purge BM contaminated with leukemia or against minimal residual disease surviving current clinical conditioning regimens and causing relapse. The agent can be used to study the role of GM-CSF receptor expressing cells in lymphohematopoiesis. It is also noteworthy that the potential of DT_{390} mGM-CSF is not limited to myeloid leukemia because high-affinity GM-CSF receptors have been detected on solid tumor cell lines including those of lung cell or colon origin.[14-17] Thus, these tumors also may be effectively targeted.

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REFERENCES

1. Gough NM, Gough J, Metcalf D, Kelso A, Grail D, Nicola NA, Burgess AW, Dunn AR: Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granolocyte-macrophage colony-stimulating factor. Nature 309:763, 1984

2. Gasson JC: Molecular physiology of granulocyte-macrophage colony-stimulating factor. Blood 77:1131, 1991

3. Chiba S, Shibuya K, Piao YF, Tojo A, Sasaki N, Matsuki S, Miyagawa K, Miyazono K, Takaku F: Identification and cellular distribution of distinct proteins forming human GM-CSF receptor. Cell Regul 1:327, 1990

4. Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A: Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): Reconstitution of a high-affinity GM-CSF receptor. Proc Natl Acad Sci USA 87:9655, 1990

5. DiPersio J, Billing P, Kaufman S, Eghtesady P, Williams RE, Gasson JC: Characterization of the human granulocyte-macrophage colony-stimulating factor receptor. J Biol Chem 263:1834, 1988

6. Park LS, Friend D, Gillis S, Urdal DL: Characterization of the cell surface receptor for granulocyte-macrophage colony stimulating factor. J Biol Chem 261:4177, 1986

7. Cannistra SA, Groshek P, Garlick R, Miller J, Griffin JD: Regulation of surface expression of the granulocyte/macrophage colony-stimulating factor in normal human myeloid cells. Proc Natl Acad Sci USA 87:93, 1990

8. Budel LM, Elbaz O, Hoogerbrugge H, Delwel R, Mahmoud LA, Lowenberg B, Touw IP: Common binding structure for granulocyte macrophage colony-stimulating factor and IL-3 on human acute myeloid leukemia cells and monocytes. Blood 75:1439, 1990

9. Park LS, Waldron PE, Friend D, Sassenfeld HM, Price V, Anderson D, Cosman D, Andrews RG, Bernstein ID, Urdal DL: Interleukin-3, GM-CSF, and G-CSF receptor expression on cell lines and primary leukemia cells: Receptor heterogeneity and relationship to growth factor responsiveness. Blood 74:56, 1989

10. Budel LM, Touw IP, Delwel R, Clark SC, Lowenberg B: Interleukin-3 and granulocyte-monocyte colony-stimulating factor receptors on human myelocyte leukemia cells and relationship to proliferative response. Blood 74:565, 1989

11. Nakamura H, Dasagawa I, Itoyama T, Mine M, Amenomori T, Sadamori N, Tomonaga M: Cytogenetic studies of non-lymphocytic neoplasias using hematopoietic growth factors. Leukemia 9:1471, 1993

12. Young DC, Griffin JD: Autocrine secretion of GM-CSF in acute myeloblastic leukemia. Blood 68:1178, 1986

13. Hoang T, Nara N, Wong G, Clark S, Minden MD, McCulloch

EA: Effects of recombinant GM-CSF on the blast cells of acute myeloblastic leukemia. Blood 68:313, 1986

14. Baldwin GC, Gasson JC, Kaufman SE, Quan SG, Williams RE, Avalos BR, Gazdar AF: Nonhematopoietic tumor cells express functional GM-CSF receptors. Blood 73:1033, 1989

15. Guillaume T, Sekhavat M, Rubinstein DB, Hamdan O, Symann ML: Transcription of genes encoding granulocyte-macrophage colony-stimulating factor, interleukin 3, and interleukin 6 receptors and lack of proliferative response to exogenous cytokines in nonhematopoietic human malignant cell lines. Cancer Res 53:3139, 1993

16. Berdel WE, Danhauser-Riedl S, Steinhauser G, Winton EF: Various human hematopoietic growth factors (interleukin-3, GM-CSF, G-CSF) stimulate clonal growth of non hematopoietic tumor cells. Blood 73:80, 1989

17. Miyagawa K, Chiba S, Shibuya K, Piao YF, Matsuki S, Yokota J, Terada M: Frequent expression of receptors for granulocyte-macrophage colony-stimulating factor on human nonhematopoietic tumor cell lines. J Cell Physiol 143:483, 1990

18. Dedhar S, Gabourv L, Galloway P, Eaves C: Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of nonhematopoietic origin. Proc Natl Acad Sci USA 85:9253, 1988

19. Foulke RS, Marshall MH, Trotta PP, Von Hoff DD: In vitro assessment of the effects of granulocyte-macrophage colony-stimulating factor on primary human tumors and derived cell lines, Cancer Res 50:6264, 1990

20. Dippold WG, Klingel R, Kerlin M, Schwaeble W, Meyer zum Buschenfelde KH: Stimulation of pancreas and gastric carcinoma cell growth by IL-3 and GM-CSF. Gastroenterology 100:1338, 1991

21. Gasson JC, Kaufman SE, Weisbart RH, Tomonaga M, Golde DW: High-affinity binding of granulocyte-macrophage colony-stimulating factor to normal and leukemic human myeloid cells. Proc Natl Acad Sci USA 83:669, 1986

22. Cheng GY, Kelleher CA, Miyauchi J, Wang C, Wong G, Clark SC, McCulloch EA, Minden MD: Structure and expression of genes of GM-CSF and G-CSF in blast cells from patients with acute myeloblastic leukemia. Blood 71:204, 1988

23. Nicola NA, Peterson L, Hilton DJ, Metcalf D: Cellular processing of murine colony-stimulating factor (Multi-CSF, GM-CSF, G-CSF) receptors by normal hemopoietic cells and cell lines. Growth Factors 1:41, 1988

24. Moya M, Dautry-Varsat A, Goud B, Louvard D, Boquet P: Inhibition of coated pit formation in Hep2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. J Cell Biol 101:548, 1985

25. Kochi SK, Collier RJ: DNA fragmentation and cytolysis in U937 cells treated with diphtheria toxin or other inhibitors of protein synthesis. Exp Cell Res 208:296, 1993

26. Yamaizumi M, Mekada E, Uchida T, Okada Y: One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell 15:245, 1978

27. Middlebrook JL, Dorland RB, Leppla SH: Association of diphtheria toxin with vero cells. J Biol Chem 253:7325, 1978

28. Lambotte P, Falmagne P, Capiau C, Zanen J, Ruysschaert JM, Dirkx J: Primary structure of diphtheria toxin fragment B: Structural similarities with lipid-binding domains. J Cell Biol 87:837, 1980

29. Murphy JR, Bishai W, Borowski M, Miyanohara A, Boyd J, Nagle S: Genetic construction, expression, and melanoma-selective cytotoxicity of a diphtheria toxin-related alpha-melanocyte-stimulating hormone fusion protein. Proc Natl Acad Sci USA 83:8258, 1986

30. Williams DP, Parker K, Bacha P, Bishai W, Borowski M, Genbauffe F, Strom TB, Murphy JR: Diphtheria toxin receptor binding domain substitution with interleukin-2: Genetic construction and properties of a diphtheria toxin-related interleukin-2 fusion protein. Protein Eng 1:493, 1987

31. Lakkis F, Steele A, Pacheco-Silva A, Rubin-Kelley V, Strom TB, Murphy JR: Interleukin 4 receptor targeted cytotoxicity: Genetic construction and in vivo immunosuppressive activity of a diphtheria toxin-related murine interleukin 4 fusion protein. Eur J Immunol 21:2253, 1991

32. Jean LF, Murphy JR: Diphtheria toxin receptor-binding domain substitution with interleukin 6: Genetic construction and interleukin 6 receptor-specific action of a diphtheria toxin-related interleukin 6 fusion protein. Protein Eng 4:989, 1991

33. Chadwick DE, Williams DP, Niho Y, Murphy JR, Minden MD: Cytotoxicity of a recombinant diphtheria toxin-granulocyte colony-stimulating factor fusion protein on human leukemic blast cells. Leuk Lymphoma 11:249, 1993

34. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR: Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61, 1989

35. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976

36. Johnson VG, Wilson D, Greenfield L, Youle RJ: The role of the diphtheria toxin receptor in cytosol translocation. J Biol Chem 263:1295, 1988

37. Lappi DA, Martineau D, Sarmientos P, Garofano L, Aranda AP, Miyajima A, Kitamura T, Baird A: Characterization of a saporin mitotoxin specifically cytotoxic to cells bearing the granulocyte-macrophage colony-stimulating factor receptor. Growth Factors 9:31, 1993

38. Waters CA, Schimke PA, Snider CE, Itoh K, Smith KA, Nichols JC, Strom TB, Murphy JR: Interleukin 2 receptor-targeted cytotoxicity. Receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells. Eur J Immunol 20:785, 1990

39. Williams DP, Snider CE, Strom TB, Murphy JR: Structure/function analysis of interleukin-2-toxin (DAB486-IL-2). Fragment B sequences required for the delivery of fragment A to the cytosol of target cells. J Biol Chem 265:11885, 1990

40. Walker F, Burgess AW: Specific binding of radioiodinated granulocyte-macrophage colony-stimulating factor to hemopoietic cells. EMBO J 4:933, 1985

41. Young DC, Wagner K, Griffin JD: Constitutive expression of the granulocyte-macrophage colony-stimulating factor gene in acute myeloblastic leukemia. J Clin Invest 79:100, 1987

42. Peleraux A, Eliason JF, Odartchenko N: Binding, internalization and degradation of radio-iodinated interleukin 3 and granulocyte-macrophage colony stimulating factor by various hemopoietic cells. Biochim Biophys Acta 1055:141, 1990

43. Rowley SD, Jones RJ, Piantadosi S, Braine HG, Colvin OM, Davis J, Saral R, Sharkis S, Wingard J, Yeager AM, Santos GW: Efficacy of ex vivo purging for autologous bone marrow transplantation in the treatment of acute nonlymphoblastic leukemia. Blood 74:501, 1989

44. Zhong RK, Donnenberg AD, Rubin J, Ball ED: Differential effect of 4-hydroperoxycyclophosphamide and antimyeloid monoclonal anitbodies on T and natural killer cells during bone marrow purging. Blood 83:2345, 1994

45. Damiani D, Michieli M, Revignas MG, Russo D, Fanin R, Michelutti A, Mallardi F, Baccarani M: Proliferation and maturation effects of in-vivo granulocyte-macrophage colony stimulating factor in acute non-lymphocyte leukemia. Haematologica 77:25, 1992

46. Woodworth TG, LeMaistre CF, McCaffrey R, Schnipper L, Rosen S, Ratain M: Phase I/II clinical studies of DAB486IL-2 fusion toxin in patients with refractory IL-2 receptor expressing malignancies. Blood 80:158a, 1992 (abstr, suppl)

47. Rudd CE, Janssen O, Cai YC, da Silva AJ, Raab M, Prasad KV: Two-step TCR zeta/CD3-CD4 and CD28 signaling in T cells: SH2/SH3 domains, protein-tyrosine and lipid kinases. Immunol Today 15:225, 1994

48. Kriegler AB, Verschoor SM, Bernardo D, Bertoncello I: The relationship between different high proliferative potential colony-forming cells in mouse bone marrow. Exp Hematol 22:432, 1994

49. Teofili L, Iovino MS, Di Mario A, Ortu La Barbera E, Pierelli L, Bussa S, Rumi C, Menichella G, Leone G: In vitro expansion of $CD34^+$ cells mobilized with chemotherapy and G-CSF. Int J Artif Organs 16 Suppl 5:89, 1993

50. Magli MC, Iscove NN, Odartchenko N: Transient nature of early haematopoietic spleen colonies. Nature 295:527, 1982

51. Baines P, Visser JW: Analysis and separation of murine bone marrow stem cells by H33342 fluorescence-activated cell sorting. Exp Hematol 11:701, 1983

52. Vitetta ES, Thorpe PE, Uhr JW: Immunotoxins: Magic bullets or misguided missiles?. Immunol Today 14:252, 1993 (review)

53. Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJ, Aglietta M, Arese P, Mantovani A: Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. Nature 337:471, 1989

1: Protein Eng. 2002 May;15(5):419-27.

Targeting glioblastoma multiforme with an IL-13/diphtheria toxin fusion protein in vitro and in vivo in nude mice.

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Fusion proteins composed of tumor binding agents and potent catalytic toxin show promise for intracranial therapy of brain cancer and an advantage over systemic therapy. Glioblastoma multiforme (GBM) is the most common form brain cancer and overexpresses IL-13R. Thus, we developed an interleukin-1 receptor targeting fusion protein, DT(390)IL13, composed of human interleu 13 and the first 389 amino acids of diphtheria toxin. To measure its ability to inhibit GBM, DT(390)IL13 was tested in vitro and found to inhibit selectivel U373 MG GBM cell line with an IC(50) around 12 pmol/l. Cytotoxicity was neutralized by anti-human-interleukin-13 antibody, but not by control antibo In vivo, small U373 MG glioblastoma xenografts in nude mice completely regressed in most animals after five intratumoral injections of 1 microg of D (390)IL13 q.o.d., but not by the control fusion protein DT(390)IL-2. DT(390 IL13 was also tested against primary explant GBM cells of a patient's excise tumor and the IC(50) was similar to that measured for U373 MG. Further stu showed a therapeutic window for DT(390)IL13 of 1-30 microg/injection and histology studies and enzyme measurements showed that the maximum toler dose of DT(390)IL13 had little effect on kidney, liver, spleen, lung and heart non-tumor-bearing immunocompetent mice. Together, these data suggest tha (390)IL13 may provide an important, alternative therapy for brain cancer.

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Targeting the over-expressed urokinase-type plasminogen activa receptor on glioblastoma multiforme.

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A recombinant fusion protein targeting the urokinase-type plasminogen activ receptor (uPAR) and delivering a potent catalytic toxin has the advantage of simultaneously targeting both over-expressed uPAR on glioblastoma cells an the tumor neovasculature. Such a hybrid protein was synthesized consisting c noninternalizing amino-terminal fragment (ATF) of urokinase-type plasmino activator (uPA) for binding, and the catalytic portion of diphtheria toxin (DT killing, and the translocation enhancing region (TER) of DT for internalizatic The protein was highly selective for human glioblastoma in vitro and in vivo vivo, this DT/ATF hybrid called DTAT caused the regression of small subcutaneous uPAR-expressing tumors with minimal toxicity to critical orga In vitro, DTAT killed only uPAR-positive glioblastoma cell lines and human endothelial cells in the form of the HUVEC cell line. Killing was selective ar blockable with specific antibody. DTAT was highly effective against tumor c cultured from glioblastoma multiforme patients and in vitro mixing experime combining DTAT with DTIL13 another highly effective anti-glioblastoma ag showed that the mixture was as toxic as the most potent immunotoxin. In this article, we review our progress to date with DTAT.

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- Review, Academic

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